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ARGONNE CANCER RESEARCH HOSPITAL
950 EAST FIFTY-NINTH STREET • CHICAGO • ILLINOIS 60637

Semiannual Report to
THE ATOMIC ENERGY COMMISSION

SEPTEMBER 1966

LEON O. JACOBSON, M.D.
Editor

MARGOT DOYLE, Ph.D.
Associate Editor

OPERATED BY THE UNIVERSITY OF CHICAGO
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CHROMOSOME STUDIES IN PRE-LEUKEMIA. I. ANEUPLOIDY OF GROUP C CHROMOSOMES IN THREE PATIENTS*

By

J. D. Rowley, R. K. Blaisdell,† and L. O. Jacobson
With the technical assistance of J. Mikuta, R. Byrne, and L. Shepley

Many patients with acute leukemia exhibit abnormalities of chromosome number or morphology in the affected (leukemic) cells which may be constant in any one patient, but may vary considerably from one patient to another.[1,2] Although it is presumed that these chromosomal abnormalities are related to the neoplastic behavior of leukemic cells,[3] their precise role in the evolution of the leukemic state remains to be determined. Direct evidence bearing on this issue would accrue from systematic analyses of chromosome constitution beginning with the presumably normal subject, progressing through the pre-leukemic phase, and culminating in the overtly leukemic stage in affected individuals. Such evidence has not been forthcoming because human acute leukemia is still a relatively rare disorder, most cases of which occur sporadically, apparently arising de novo, and usually not recognized until clinically manifest. However, the occurrence of certain hematologic syndromes with a propensity to terminate in acute leukemia,[4] affords an opportunity to assess the importance of chromosomal alterations in the pathogenesis of leukemia. These pre-leukemic or, more precisely, potentially leukemic syndromes, may be classified into the aplastic, myelodysplastic, and myeloproliferative disorders. Although the first and the third of these terms are conventional designations requiring no further elaboration, the myelodysplasias[4,5] form a group of disorders exhibiting morphologic evidence in the marrow and the blood of disturbed formation of any, or any combination, of the major blood cell lineages (erythroid, granulocytic, megakaryocytic), not characteristic of the aplastic, the myeloproliferative, or the leukemic states, yet displaying features often intermediate or transitional between these conditions. The following examples illustrate the heterogeneity of this group and yet its separateness from the aplastic, myeloproliferative and leukemic classes: refractory hyperplastic anemia, sideroachrestic anemia, atypical myeloid disorder, pre-leukemic acute leukemia, primary splenic hematopenia. Although certain causal factors might be implicated, the etiology in most cases remains obscure, and most cases are fatal or terminate after conversion to acute leukemia.

Only five cases of persistent abnormalities of chromosome morphology have been reported in patients with potentially leukemic disorders, in whom leukemic transformation has not occurred;[6-10] in all other cases with abnormal karyotypes, leukemia was evident when the marrow was first examined.[6,8,11]

During the investigation of 15 potentially leukemic patients who manifested obscure anemia, leukopenia, thrombocytopenia or thrombocythemia, we have found three individuals with abnor-

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mal chromosome numbers, none of whom has yet developed acute leukemia. In each case, the abnormality is confined to the C group chromosomes, but it is a different alteration in each instance. The respective diagnoses have been: aplastic anemia, idiopathic sideroachrestic anemia, and idiopathic thrombocythemia—each being an example of the three classes of potentially leukemic hematologic syndromes referred to above. This is the first report of chromosome abnormalities in sideroachrestic anemia and thrombocythemia. A case of aplastic anemia with 47 chromosomes has been reported, although no karyotypic analysis was included.[12]

Since these conditions may all terminate in leukemia, it seems likely that the existence of such chromosomal abnormalities before the onset of overt leukemia should be considered in any hypothesis relating aneuploidy and leukemia. Our purpose in presenting these cases at this time is to examine the possible significance of the chromosomal alteration and to encourage other investigators to report similar cases.

MATERIALS AND METHODS

Chromosome preparations were made using a modification of the direct bone marrow technique of Tjio and Whang.[13] The bone marrow sample of approximately 1 ml was put in a centrifuge tube containing 5 ml of Hank's solution to which was added 0.1 ml of commercial heparin (1000 units/ml) and 0.1 ml of an 0.5 μg/ml solution of vincaleukoblastine (Velban, Lilly). After thorough mixing, the sample was incubated for 1 hour at 37° C and centrifuged at 600 rpm for 10 minutes; the supernatant was discarded, and 8 - 10 ml of warm (37° C) hypotonic (0.95 per cent) sodium citrate solution was added. The material was incubated for 20 minutes at 37° C, centrifuged for 10 minutes at 600 rpm, and the supernate was discarded. The cells were gently resuspended in 5 ml of fresh 1:3, glacial acetic acid:absolute methanol, fixative. After 45 minutes, the cell suspension was centrifuged, the first fixative supernatant was removed, and the cells were resuspended in 5 ml of 45 per cent glacial acetic acid and 55 per cent methanol. This second fixative was changed twice daily until chromosome preparations of suitable quality were obtained. This occasionally took over one week, and required more than a dozen changes of fixative. The material was stored at -4° C during this period. Slide preparations of the cells were then made by the standard air-dry technique;[14] they were stained with Leishman-Giemsa stain and mounted in Histoclad (Clay-Adams, Inc.).

Peripheral blood was cultured using essentially the technique of Moorhead.[15] The leukocytes were separated from the red cells by centrifuging the heparinized blood at 600 rpm for 10 minutes. The buffy coat was removed and added to TC medium 199, with phytohemagglutinin M (Difco). Velban was added either overnight or for 2 hours before terminating the culture, which was usually after about 72 hours.

Buccal smears were prepared according to a modified technique of Ross.[16]

CASE REPORTS

Case 1

A 23-year-old white, unmarried, male spray-painter was apparently well until November 1963, when he first noted furuncles of the buttocks. Chloramphenicol orally and intramuscularly was prescribed, totaling approximately 16.0 g during a 3-week period. The infection subsided, but in January 1964, he began to have frequent epistaxis, easy bruising, and progressive weak-

ness. In April 1964, a second course of chloramphenicol, 3.0 g over 4 days, was given for a sore throat.

As a painter, the patient had used lacquer and various thinners for the previous three years. No hematological examinations had previously been performed.

There was no family history of a similar illness, other hematologic disorders, or developmental anomalies.

When seen at the University of Chicago Hospitals on May 15, 1964, physical examination revealed pallor and petechiae over the extremities. There was no palpable enlargement of the lymph nodes, the spleen or the liver. He had normal adult external genitalia and hair distribution.

Laboratory examination disclosed hematocrit 21 per cent, hemoglobin 6.8 g per 100 ml, reticulocytes 0.1 per cent, total leukocytes 3,000 per cu mm, with segmented neutrophils 19 per cent, lymphocytes 77 per cent, and monocytes 4 per cent; and platelets 4,400 per cu mm. The granulocyte alkaline phosphatase score was 81 (normal range 15 to 50). The red cells were Xg (a+). Assays of hemolysates for glucose-6-phosphate dehydrogenase (G-6-PD) activity were within normal limits; starch gel electrophoresis revealed the normal G-6-PD B pattern.

Sternal bone marrow sections showed pronounced hypocellularity, with substantial reduction of granulocytes, erythroid precursors, and megakaryocytes, and replacement of these elements by fatty tissue. No abnormal cells were seen. Excessive stainable iron was demonstrated.

The pancytopenia had failed to respond to iron, liver extract, folic acid, and vitamin B-12 therapy during April 1964 and only transient benefit had been noted after four separate blood transfusions.

In September 1964, after four months of oral fluoxymesterone (Halotestin) 40 mg daily, the patient's hemoglobin value rose to 15.9 g per 100 ml. In February 1965, another bone marrow examination, seven months after the preceding one, revealed moderate erythroid activity, a persistent paucity of myeloid cells and megakaryocytes, but no evidence of leukemia.

When most recently seen on May 14, 1965, the patient had married and returned to work as a lift machine operator. There was still no palpable enlargement of hemopoietic organs, but recent purpuric lesions were evident on his limbs. The patient's hemoglobin level has remained normal in spite of a reduction in androgen dosage to 10 mg daily. However, a moderate leukopenia has persisted (white cell count 3200 per cu mm) with normal differential percentages; the severe thrombocytopenia (platelets 7,700 per cu mm) has not improved significantly.

The diagnosis has been aplastic anemia (marrow panhypoplasia with pancytopenia) probably related to chloramphenicol and/or organic solvents, with partial remission, induced by androgen therapy.

Case 2

This 62-year-old man was found to have a severe refractory anemia in July 1961. In November 1961 his spleen was removed; it weighed 746 g and exhibited "hyperemia" microscopically. This procedure failed to relieve the anemia. A total of 17 units of blood (approximately 4.25 g of iron) were given over a 4-year period. In January 1963, mental deterioration, hepatomegaly, and diabetes mellitus were noted.

Family history included no known similar or related illnesses.

Although the patient had been married twice, each wife had had a hysterectomy prior to

marriage, and thus, there were no children.

Examination at the time of admission to the University of Chicago Hospitals on November 1, 1964 revealed mental confusion, brown-grey dermal pigmentation, moderate enlargement of the heart, and pedal edema. The liver was slightly enlarged, but there were no palpable lymph nodes. The external genitalia were of normal size and form.

Blood examinations disclosed hemoglobin 6.5 g per 100 ml, hematocrit 20 per cent, red cell count 1.80 x 10^6 per cu mm, mean cell volume 110 cu μ, mean cell hemoglobin concentration 32 per cent and reticulocyte count 4.6 per cent. The total leukocyte count was 8,200 per cu mm (corrected for normoblastemia), with segmented neutrophils 44 per cent, lymphocytes 44 per cent, monocytes 10 per cent, eosinophils 1 per cent, and basophils 1 per cent. The platelets numbered 252,000 to 607,200 per cu mm. Red cell aniso-poikilocytosis, hypochromia, iron-positive inclusions, target cells and 94 nucleated erythrocytes per 100 white cells were seen in the blood smears.

Sternal marrow material aspirated in November 1964 showed intense erythroid hyperplasia, with a predominance of early erythroid precursors and an abundance of normoblasts with stainable iron inclusions ("ring sideroblasts"). There was no morphologic evidence of disturbed granulopoiesis or altered megakaryocyte-platelet formation. Stainable iron was pronounced and electron microscopy revealed heavy aggregates of iron in the mitochondria of marrow normoblasts, which is considered to be characteristic of certain sideroblastic anemias.[17] A second marrow examination on April 20, 1965, just prior to death, revealed no significant change.

Death followed heart failure, atrial fibrillation, and pneumonia with septicemia. The clinical diagnosis was idiopathic sideroachrestic anemia, with progression to hemochromatosis involving the liver, pancreas, skin, and heart.

Autopsy confirmed the clinical diagnoses and revealed no evidence of leukemia. Histologic examination of the testes showed hyalinization of the basement membrane of the tubules, and absent spermatogenesis consistent with chronic liver disease; but no prominence of Leydig cells to support a diagnosis of the Klinefelter syndrome.

Case 3

This 59-year-old Negro housewife first noted prolonged bleeding following dental extractions in 1945. Since 1956, she had had four episodes of upper abdominal distress with melena, requiring blood transfusion, and in 1960, x-ray examination revealed a duodenal ulcer.

No similar illness or hematologic disorder was known in the family.

The patient had a normal menstrual history with menopause at age 49. Although she had become pregnant on four occasions, spontaneous abortions with profuse uterine hemorrhage had occurred within a few months of the beginning of each pregnancy.

Physical examination, when the patient was first seen at the University of Chicago Hospitals in January 1964, disclosed no deformities, normal adult external genitalia, but a somewhat small cervix and uterine corpus. Neither the liver, spleen, nor lymph nodes were palpably enlarged. There were no external evidences of hemorrhage and no signs of vascular occlusive disease.

Laboratory examinations showed a hemoglobin value of 10.4 g per 100 ml, hematocrit 34 per cent, mean cell volume 77 cu microns, mean cell hemoglobin concentration 31 per cent, reticulocytes, 1.9 per cent, plasma iron 85 μg per 100 ml and unsaturated transferrin 300 μg

per 100 ml. In the blood smear, the red cells were hypochromic. Total leukocytes numbered 10,100 per cu mm, with neutrophils 76 per cent, lymphocytes 15 per cent, monocytes 3 per cent, and eosinophils 6 per cent. Platelet counts varied from 667,000 to 1,375,000 per cu mm. Granulocyte alkaline phosphatase scores ranged from 39 to 104.

Sternal marrow sections revealed normal overall cellularity, although megakaryocytes and platelets were unusually prominent. Stainable iron was reduced.

Following iron administration, the patient's hematocrit rose to a high of 55 per cent and on one occasion a peak red cell volume measurement of 38.4 ml per Kg (normal 23 to 36 mg per Kg) was recorded.

After the three marrow examinations, busulfan (Myleran) was prescribed in oral doses of 2 - 8 mg daily; but only after one month did the blood platelet counts fall to the 200,000 to 300,000 per cu mm range.

The diagnosis has been idiopathic thrombocythemia, but the elevated hematocrit and red cell volume value on one occasion after iron therapy permits consideration of the diagnosis of polycythemia vera, masked by chronic bleeding and iron deficiency.

CHROMOSOME STUDIES

Case 1

Analysis of the marrow chromosomes revealed two cell populations (Table 1). In the first bone marrow specimen, 5/58 cells had 46 chromosomes and an apparently normal karyotype, whereas 36/58 cells had 45 chromosomes (Figure 1). Thirteen of the hypodiploid cells were analyzed in detail and one group C chromosome was consistently missing from each metaphase. Nine of the 16 metaphases that contained less than 45 chromosomes were karyotyped and each of these cells was consistently lacking one C group chromosome, in addition to one or more other chromosomes, apparently on a random basis.

In contrast, the majority of cells from the peripheral blood had 46 chromosomes, and a normal karyotype. Hypodiploid cells showed random loss of one or more chromosomes, presumably due to cell breakage.

Similar results were obtained on analysis of the second bone marrow specimen, aspirated seven months later. However, only 1/59 metaphases contained 46 chromosomes, whereas 5/58 chromosomes had appeared normal in the first sample. Seventeen of the 46 metaphases containing 45 chromosomes were analyzed in detail, and again showed the consistent loss of a C group chromosome. Analyzed cells from the second peripheral blood specimen had 46 normal chromosomes with one exception. This cell had 48 chromosomes; chromosome morphology was too poor to karyotype with certainty, but the cell appeared to have two extra small chromosomes belonging to groups E, F, or G-Y.

The proportion of hypodiploid cells in both bone marrow samples and the first peripheral blood specimen is much higher than normal for our laboratory. This may reflect some increased fragility of the patient's mitotic cells.

Case 2

The modal chromosome number in metaphases from both direct bone marrow preparations was 47 (Table 1). The extra chromosome was medium-sized, submetacentric, and belonged to the C group (Figure 2). In the first sample, of the 6 cells containing 46 chromosomes, none was

Table 1

CHROMOSOME ANALYSES IN THREE PATIENTS

Case No.	Date	Source	Chromosome number					Other	Total	Comment
			<45	45	46	47	48			
1	7/24/64	Marrow	16(9)	36(13)	5(5)			1 cell-91(1)	58(28)	One C chromosome missing
	9/28/64	Blood	4(1)	1(1)	20(4)				25(6)	
	2/16/65	Marrow	11(4)	46(17)	1(1)			1 cell-92(1)	59(23)	
	2/16/65	Blood	0	0	14(7)		1(1)		15(8)	
2	1/12/65	Marrow	3(3)	5(5)	6(5)	37(18)	2(2)	1 cell-68(1)	54(34)	One extra C chromosome
	3/5/65	Blood	1(1)	4(2)	40(17)	2(2)	1(1)	1 cell-49(1)	49(24)	
	4/26/65	Marrow	8(0)	6(1)	14(11)	42(11)	1(1)	1 cell-45-46 4 cells-46-47	76(24)	
3	1/15/65	Marrow	1(1)	5(4)	27(19)	3(3)	10(9)		46(36)	Two extra C chromosomes
	1/15/65	Blood	1(0)	5(4)	17(12)				23(16)	
	3/29/65	Marrow†		1	1			3 cells-46-47(3)	5(3)	
	4/14/65	Marrow	1(1)	1(1)	2(2)	1(1)		1 cell-<46(1)	6(6)	
	4/14/65	Marrow†	7(4)	5(3)	13(8)	1(1)	4(4)	1 cell-45-46(1) 3 cells-46-47(3)	34(24)	

( ) Number in parentheses indicates number of photographed cells analyzed in detail.

†Six-hour incubation with ^{3}H thymidine.

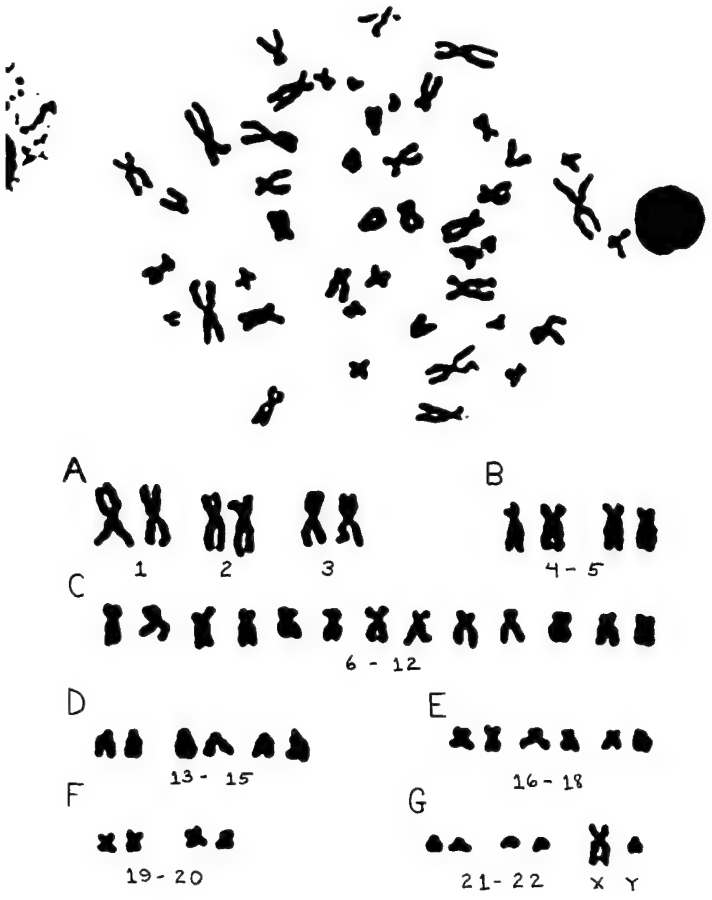

Figure 1. Case 1, aplastic anemia. Above: metaphase plate from bone marrow, photographed with bright field illumination. Below: karyotype of the same cell containing 45 chromosomes, with one C group chromosome missing.

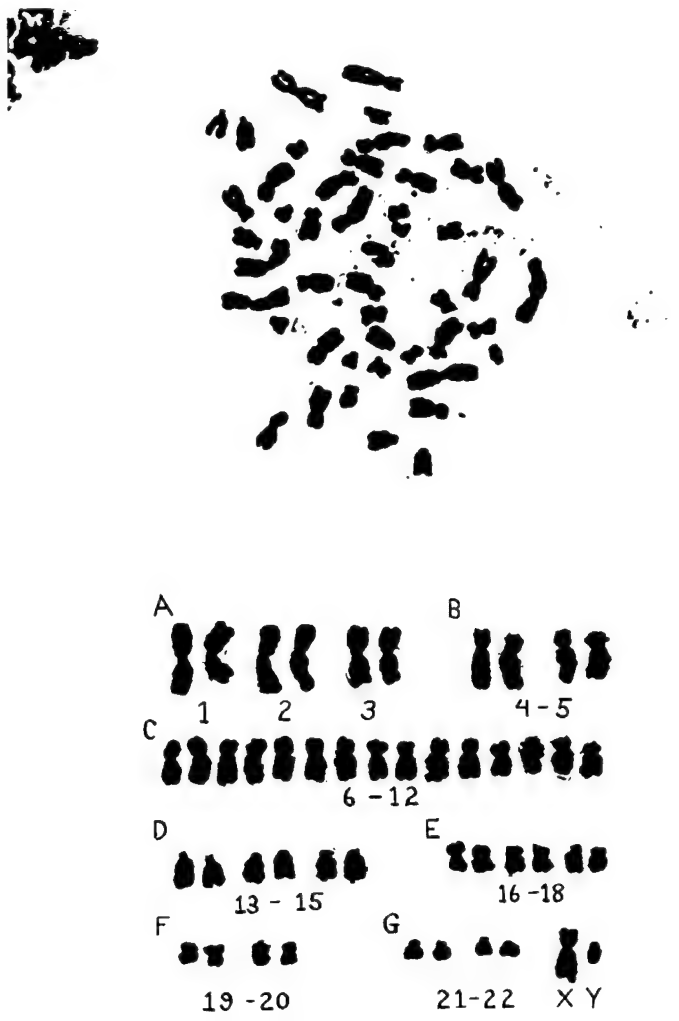

Figure 2. Case 2, sideroachrestic anemia. Above: metaphase plate obtained from direct bone marrow preparation, photographed with bright field illumination. Below: karyotype of the above cell showing 47 chromosomes with one extra C group chromosome.

clearly normal; 3 cells had an extra C and were missing an E chromosome, 2 cells had an extra C and were missing a G, and one cell could not be karyotyped because of poor morphology. In the second sample, three months later, of the 11 analyzed cells with 46 chromosomes, 5 had the normal number of chromosomes in the C-X group. These 5 could be normal cells or, as seems quite likely, they could be the result of breakage of a 47 chromosome metaphase with loss of a C group chromosome. Each of the other 6 analyzable cells with 46 chromosomes had an extra C chromosome; 1 was lacking an A chromosome, 1 was lacking a D, 2 were lacking an E, and 2 were lacking G-Y chromosomes. One of five metaphases with 45 chromosomes was lacking an F and G chromosome but had an extra C chromosome. The other 4 metaphases were lacking both a C plus some other chromosome (assuming they resulted from breakage of the hyperdiploid line). In the first sample, 2 metaphases had 48 chromosomes with 2 extra chromosomes in the C group. In the second preparation, however, the 1 metaphase with 48 chromosomes had an extra C and an extra E. This would suggest that the cells with 48 chromosomes were the result of mitotic accidents, such as non-disjunction, rather than representatives of an additional stable hyperdiploid stem line.

Forty out of 49 cells obtained from a 72-hour peripheral blood culture contained 46 chromosomes. Seventeen of these were karyotyped and revealed normal chromosomes. Cells with less than 46 chromosomes showed random loss as a result of breakage. The metaphases with more than 46 chromosomes were of poor morphology, but the extra chromosomes appeared to be small and could belong to group F, or G-Y, or could be fragments. No metaphases with a karyotype similar to the hyperdiploid line found in the marrow were obtained from the peripheral blood. Two 6-hour bone marrow cultures with ^{3}H thymidine have been done; the results are not yet available. Two separate skin cultures have been established, but the results are not yet available. Buccal smears were negative for sex chromatin (Barr) bodies.

Case 3

Two distinct cell lines were present in the two bone marrow specimens (Table 1). In the first sample, 27 of 46 metaphases contained 46 chromosomes and had an apparently normal karyotype. Six cells were hypodiploid with random loss of one or more chromosomes due to breakage of diploid cells. Nine of 46 metaphases had 48 chromosomes with two extra chromosomes in group C (Figure 3). Metaphases with 47 chromosomes probably resulted from breakage of this latter cell line, as one metaphase had 2 extra C's and was without a D chromosome; the other 2 cells with 47 chromosomes had 1 extra C chromosome. The same two stem-lines were present in the second bone marrow preparation, but the proportion of hyperdiploid metaphases was lower. This might be related to the 6-hour culture period used to study the ^{3}H thymidine labeling pattern of these chromosomes. As Sandberg and colleagues have shown[18] aneuploid bone marrow cells do not tolerate in vitro culture as well as diploid cells do. The results of the ^{3}H thymidine labeling are not yet available.

No hyperdiploid cells were found in the peripheral blood culture. Most hypodiploid cells had resulted from random loss of chromosomes from diploid cells. Detailed analysis of the cells with 46 chromosomes revealed that 5 of 17 cells in the peripheral blood had inconsistent abnormalities in the karyotype. In two such cells there appeared to be two extra C chromosomes, replacing a D and an F in one cell, and an E and a G in the other cell. These two cells would appear to be related to the cells with 48 chromosomes found in the marrow; however, since no

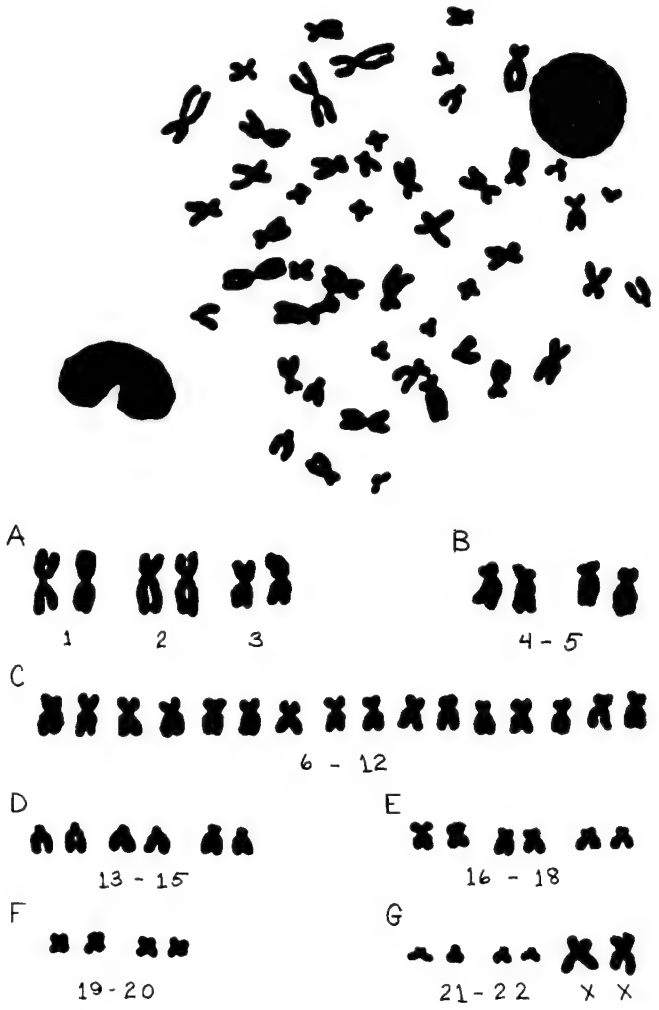

Figure 3. Case 3, idiopathic thrombocythemia. Above: metaphase plate obtained from a direct bone marrow preparation, photographed with bright field illumination. Below: karyotype of the same cell containing 48 chromosomes with 2 extra C group chromosomes.

hyperdiploid cells have been found in the peripheral blood, this apparent relationship seems doubtful. Of the other 3 abnormal yet diploid cells, one appeared to have 7 A group chromosomes while lacking a C; the second seemed to have an extra C in place of an E, and the third had an extra G replacing a D group chromosome. Buccal smears showed only 1 sex chromatin mass in 27 per cent of 200 cells counted.

DISCUSSION

Our three patients presented with apparently unrelated hematologic syndromes, yet all three of them had marrow chromosomal abnormalities involving the C group. These cases emphasize the need for including a study of the bone marrow, as well as of the blood, in any comprehensive cytogenetic examination of hematologic disorders. All three patients showed persistent chromosomal aberrations in some or all metaphases from the bone marrow; and all had

essentially normal karyotypes in metaphases from the peripheral blood. Because of the predominantly diploid pattern of the lymphocytes cultured from the peripheral blood, the chromosomal abnormalities described are confined to marrow cells and have developed after embryogenesis, that is, they are presumably acquired and not congenital.

In Case No. 1 (aplastic anemia with 45 chromosomes) an autosome in the C group has been lost. The presence in the patient's red blood cells of the Xg^a antigen[19] and a B band for G-6-PD[20] which are X-linked indicate that the patient does have an X chromosome and thus excludes the possibility that the lost C chromosome is an X chromosome. The karyotype of the hypodiploid cells in the bone marrow has remained constant over a 7-month period; indeed, the proportion of hypodiploid cells apparently has increased at the expense of normal diploid cells. Clinically the patient's anemia has responded to androgen therapy, although his white blood cell and platelet counts remain low. The chromosome changes are probably not specific for chloramphenicol marrow toxicity, for we have found a normal marrow karyotype in a woman who developed pancytopenia after exposure to chloramphenicol.

With regard to Case No. 2 (sideroachrestic anemia with 47 chromosomes) and Case No. 3 (idiopathic thrombocythemia with 48 chromosomes), our present interpretation is that they are examples of autosomal trisomy. The alternative diagnoses of Klinefelter's syndrome for Case No. 2, and quadruple -X female for Case No. 3, seem unlikely for the following reasons: the majority of cells in the peripheral blood have 46 chromosomes, the external genitalia are normal and the buccal smears are compatible with normal male and normal female sex chromosome complements respectively. Autoradiography is being used to determine the number of late-labeling X chromosomes in Cases No. 2 and 3.[21] If our diagnoses are correct, Case No. 2 should have no late-labeling X chromosome and Case No. 3 should have only one.

In the five cases of group C trisomy that have been previously reported,[22-26] the abnormality apparently occurred in the developing zygote. All were mosaics, with some cells containing 46 normal chromosomes, and a variable proportion of metaphases that had 47 chromosomes, with trisomy in the C group. The chromosome abnormality was observed in cells from the blood and/or skin; there was no mention of a bone marrow examination in any of these cases. These patients had developmental anomalies of the skeleton, nervous system, and/or genitalia, and no hematologic abnormality, with the exception of one woman who was normal, but whose children in 2 of 3 pregnancies had multiple, severe, congenital abnormalities.

The majority of the reported cases of aneuploidy for group C chromosomes have involved patients with leukemia, especially acute leukemia, although patients with chronic myelogenous leukemia may also show chromosomal abnormalities in addition to the Ph^1 chromosome.[27] A review of the following papers is given, since in most of these presentations there is one case with a stable karyotypic abnormality occurring in a potentially leukemic patient allegedly "without leukemia." In Nowell and Hungerford's report[6] of 7 cases of myeloproliferative disorders, 4 patients showed marrow chromosomal aberrations. Two, or possibly three, patients whose illness had converted to subacute granulocytic leukemia, had abnormalities involving C group chromosomes. The first patient, with polycythemia converted to granulocytic leukemia, had 47 chromosomes with trisomy C and deletion of a D chromosome, yielding one resembling a G chromosome. In the second case, diagnosed as myeloid metaplasia which "converted to subacute granulocytic leukemia," one missing E group chromosome was replaced by a chromosome resembling a C group chromosome. The third case was a patient with polycythemia which had con-

verted to myelofibrosis, but with blood counts suggestive of leukemia; the modal chromosome number was 45 with chromosomes missing from either the C or E group. The fourth, a patient with polycythemia vera who had received extensive radiophosphorus therapy and x-radiation to the spleen, had a modal chromosome number of 46, but all six analyzed bone marrow cells showed a deletion of the long arm of a D chromosome resulting in an apparent extra chromosome in the G group. This patient is the only one in whom there were no signs of leukemia.

Sandberg and colleagues studied 20 patients with myeloproliferative disorders "other than leukemia" and found only one patient with a chromosomal aberration.[11] The karyotypic abnormality occurred in an unusual case of myeloid metaplasia with "a possible leukemic-like terminal phase" and was considered to be trisomy of C 9. Forty-seven chromosomes were found in both bone marrow and peripheral blood cells on the initial examination, and in the bone marrow on a second examination. Sandberg and collaborators, in discussing their patients, as well as those of Nowell and Hungerford, concluded that "chromosomal abnormalities were seen predominantly in patients with a terminal leukemic stage of the myeloproliferative disorder."

Goh and Swisher found no consistent morphological chromosomal abnormalities in their 8 cases of myeloid metaplasia.[28] Solari's group[7] described a patient with myeloid metaplasia following polycythemia vera, who had been treated with x-radiation to the large bones and spleen, as well as radiophosphorus, and whose marrow cells showed a consistent replacement of one C group chromosome by an abnormally short acrocentric-type of chromosome. It was their impression that the abnormal chromosome resulted from the deletion of most of the long arm of one C group autosome. This patient had an elevated white blood count of 42,800 with 12 per cent myelocytes, and 5 per cent metamyelocytes, present in the differential examination. Although no blasts were reported, these blood cell findings raise the possibility of leukemia. It is also conceivable that the chromosomal change was related to ionizing radiation. That irradiation can produce chromosomal aberrations is well know.[29] These aberrations consist of achromatic gaps, breaks, fragments, dicentrics, and rings, which occur sporadically in a variable percentage of cells. The consistent karyotypic abnormality reported by Nowell and Hungerford, and by Solari and his co-workers in their irradiated patients may represent a stem-line which evolved from these aberrant cells or may be entirely unrelated to the patient's radiation therapy.

Freireich and associates[8] reported three cases of refractory anemia, thrombocytopenia and marrow granulocytic hyperplasia, in which the marrow cells had only 45 chromosomes and one missing C group chromosome, whereas cultured peripheral blood and skin cells showed 46 chromosomes. Two of the three patients died of "acute myelomonocytic leukemia." These patients resemble our Case No. 1 with regard to cytogenetic findings, anemia, and thrombocytopenia, but our patient had a very hypocellular marrow.

Kiossoglou and Mitus,[9] in a recent abstract describing chromosomal studies in chronic myeloproliferative syndromes, report that 14/14 patients with polycythemia vera and 2/2 with thrombocythemia had normal marrow karyotypes; 1/3 patients with myelofibrosis-myeloid metaplasia showed hyperdiploidy with an extra C group chromosome. Kemp[10] has followed a patient for four years whose clinical diagnosis is polycythemia vera; a significant number of cells in her bone marrow have 48 chromosomes with 2 extra chromosomes in the C group, whereas peripheral blood cells and cells from fibroblast cultures contain the normal diploid number. She has shown no sign as yet of leukemic transformation.

If one considers only the five patients in the above-mentioned reports (Table 2) who, to date,

Table 2

SUMMARY OF CHROMOSOME CONSTITUTION OF MARROW AND BLOOD CELLS IN CASES OF MYELOPROLIFERATIVE SYNDROME WITHOUT OVERT LEUKEMIA

Age	Sex	Source	Chromosome number					Other	Karyotype analysis		Comments	Reference
			<45	45	46	47	48		Normal	Abnormal		
36	F	Marrow† (2)		6	28				0	6 Deletion of long arm of D → G	Polycythemia vera; Rx-P-32 and x-radiation to spleen; mild thrombocytopenia	Nowell[6]
		Blood† (2)		3	14			5 cells < 47	1	1 Fragments		
54	M	Marrow	2	2	45				0	7 Replacement of C chromosome by a short acrocentric, probably deletion of most of long arm of C group autosome	Polycythemia vera; Rx-x-ray to bone and spleen; P-32; WBC, 42,800; myelocytes, 12 per cent; metamyelocytes, 5 per cent	Solari[7]
		Marrow Blood and Skin		*	*			Data not given in abstract		One C chromosome missing	Refractory anemia, thrombocytopenia, granulocytic hyperplasia of bone marrow	Freireich[8]
								Data not given in abstract		One extra C chromosome	Myelofibrosis-myeloid metaplasia	Kiossoglou[9]
	F	Marrow Blood and Skin			*		*			Two extra C chromosomes	Polycythemia vera	Kemp[10]
23	M	Marrow† (2)	27	82	6			2 tetraploid	6	30 One C chromosome missing	Pancytopenia, hypocellular marrow	Present report, Case No. 1
		Blood† (2)	4	1	34		1		11	1 Two extra small chromosomes		
62	M	Marrow† (2)	11	11	20	79	3	6	5**	29 One extra C chromosome	Sideroachrestic anemia	Case No. 2
		Blood	1	4	40	2	1	1	17	4 One to 3 extra small chromosomes or fragments of chromosomes		
59	F	Marrow† (3)	9	12	43	5	14	8	29	13 Two extra C chromosomes	Idiopathic thrombocythemia	Case No. 3
		Blood	1	5	17				12	5** Inconsistent abnormalities		

*Exact figure not given; indicates modal number stated in report.

**See discussion in text.

†Combined results of several examinations all of which yielded similar results. Numbers in parentheses indicate number of studies.

have shown no sign of overt leukemia, 4 out of the 5 show abnormalities of C group chromosomes involving either deletion of most of the long arms of a C, monosomy, trisomy, or tetrasomy (or, alternatively a double trisomy) for a C chromosome. Only one of Nowell and Hungerford's cases involved a chromosome of another group, viz., deletion of a portion of the long arms of a D chromosome which resulted in a chromosome resembling a G group autosome.

The addition of our three cases, each showing a different abnormality, but all involving a C group chromosome, reveals the remarkable association of aberrant C chromosomes with a diversity of hematologic disorders, many of which terminate in acute leukemia.

Reisman and co-workers have published a careful study of eight cases of acute stem-cell leukemia in children in which serial observations were made during repeated relapses and intervening remissions.[3] During remission, the normal diploid mode of 46 in marrow cells was invariably restored, regardless of the duration of the disease or the therapy employed. All patients, untreated or in relapse, had hyperdiploid modal numbers ranging from 47 to 65. In the second or third relapses studied, the originally observed aneuploid karyotype always reemerged after remissions of varying lengths up to four months. They concluded that a change in the chromosomal constitution of the malignant cell is one of the basic alterations in acute leukemia and is present in the earliest phases of the disease that can be investigated.

Our patients have changes in the chromosome constitution of marrow cells similar to those found in acute leukemia, and thus a crucial question to be asked about our two surviving patients is, will they develop leukemia? How long can a cell line be maintained with an abnormal genetic constitution which apparently continues to respond to homeostatic control mechanisms? If a patient can live for more than seven months (as in Case No. 1) or for more than four years[10] with a large proportion of bone marrow cells having an aneuploid number, then it would seem that a stable aneuploid cell line is not of itself sufficient for the development of leukemia.

On the other hand, Engel et al.'s report of 7 infants with Down's syndrome (mongolism) with transient congenital leukemia[30] suggest that aneuploidy and leukemia may be reversible in rare instances. One child was considered to have acute myelogenous leukemia diagnosed on the tenth day of life on the basis of hepatosplenomegaly, ecchymoses, white blood cell count of 34,400 with 36 per cent blasts, and a platelet count of less than 10,000. Bone marrow examination showed 40 per cent granulocytes and 33 per cent primitive mononuclear cells. The modal chromosome number in a direct marrow preparation was 57. A complete hematologic remission without therapy was noted in three months and the child was still well at three years of age. Marrow chromosome examination in remission revealed 47 chromosomes with trisomy 21 which is "usual" for Down's syndrome. Karyotyping was not done on the other 6 cases during the initial "leukemic" phase; 4 of the children were examined in remission and had the standard trisomy 21.

Our patients are clinically quite dissimilar, one having aplastic anemia possibly due to chloramphenicol, the second, idiopathic sideroachrestic anemia, and the third, idiopathic thrombocythemia. All three conditions are related in their predisposition to terminate in acute leukemia,[4,31,32] a predisposition which also characterizes the 5 cases reported by others and summarized above.

It is possible that a leukemogenic agent (e.g., virus, radiation or chemical carcinogen) acts initially to alter the genetic constitution of the cell. This altered genome may be reflected in a gross change in the chromosomes which results in the gain or loss of a chromosome or in the deletion of part of a chromosome. A genetically significant chromosomal deletion could occur

which would be undetected by our current, relatively insensitive techniques. However, although our data suggest that an altered chromosome number does not of itself denote a neoplastic change, a stable aneuploid stem line may provide a more propitious milieu for the original agent or a different agent to transform abnormal, but still partially controllable, cells to abnormal but autonomous cells.

The significance of the observation that all 3 of our cases, plus 4 of the 5 cases of others, involve abnormalities of group C chromosomes may now be considered. Patau[33] has shown that the variation in size between known homologous chromosomes may be greater than the difference in size between adjacent pairs of chromosomes in group C. Because of the uncertainties attending the exact identification of group C chromosomes, specific identification of the missing C chromosome in Case No. 1 and the duplicated C chromosome(s) in Cases 2 and 3 is not possible at present: the same chromosome may be involved in all three cases. It is also worth noting that many cases of aneuploidy in acute leukemia involve C group chromosomes.[1] In part, this probably results from the larger number of chromosomes present in the C group. It is also possible that genes responsible for the homeostatic control of hemopoietic tissue are located on one or more C group chromosomes. Any alteration in the number of C group chromosomes, whether by an increase or a decrease, may change the genetic balance of the system and thus disturb the normal homeostatic control of hemopoiesis. This would account for the observations that deletion as well as duplications of C group chromosomes seem to be associated with hematologic disorders, many of which terminate in acute leukemia. However, the determination of the possible relationship between C group aneuploidy and acute leukemia and related hematopoietic disorders depends on the accumulation of more case reports. Much valuable information could be obtained from the publication of similar cases of chromosome abnormalities observed in patients without overt signs of malignant neoplasia.

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THE REGULATION OF NUMBERS OF PRIMITIVE HEMOPOIETIC CELLS*

By

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It is generally accepted that blood cells are derived from primitive undifferentiated cells in the bone marrow which are usually called stem cells. Morphological characterization of stem cells is impossible on other than arbitrary, authoritarian grounds, since they can be identified morphologically only by the absence of any feature characteristic of one of the many cell lines giving rise to mature blood cells. Investigations of stem cells dependent on morphological features therefore appear destined to be limited, and suspect, and in recent years, their functional characteristics have come under investigation. These studies have all been consequences of the monumental experiments of Jacobson et al.[1] in which animals receiving otherwise lethal doses of ionizing radiation survived, provided that the spleen was shielded from injury. It is likely that the mechanism underlying this survival is the recolonization of the system by primitive cells carried in the blood stream from the protected spleen.[2,3] A host of radiobiological investigations culminating in the classic demonstration of spleen cloning, have been direct outgrowths of these experiments. McCulloch and Till[4] demonstrated that stem cells, or colony forming units, may be assayed by the number of discrete clones demonstrable in the spleens of lethally irradiated mice ten days after irradiation. Over a wide range, the number of clones is proportional to the number of normal bone marrow cells injected after irradiation.

Another method of investigating primitive marrow cells has been developed in our laboratory.[5] Since morphological evidences of erythropoiesis disappear in the transfusion-induced plethoric mouse, and are manifest again after a single injection of erythropoietin, it was concluded that erythropoietin acts upon stem cells, inducing differentiation into the erythroid line.[6] This conclusion had previously been reached independently by Alpen and Cranmore[7] and Erslev[8] who employed different models for their studies. Using the response to a standard dose of erythropoietin as a challenge to the primitive cells of the hematopoietic tissue, it has been possible to quantitate damage and follow patterns of recovery of these cells in situ and in vivo.[5,9-11]

Bruce and McCulloch[12] have recently concluded that the primitive marrow cell responsive to erythropoietin is different from the primitive marrow cell capable of proliferating to form a colony after transplantation into the lethally irradiated mouse. The present report describes experiments which indicate that both erythropoietin-sensitive cells (ERC) and colony-forming units or cells (CFU) constitute compartments of cells whose numbers are regulated. This conclusion is based upon evidence interpreted by us as indicating that the rate of proliferation of small pro-

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tected groups of these cells and their progeny increases after most of these cells in the body have been destroyed by irradiation.

METHODS

CF No. 1 female mice 12 to 14 weeks of age were used throughout. Rockland Mouse/Rat Diet and water were permitted ad libitum. X-radiation was administered by a 250,000 volt deep x-ray unit with 0.25 mm Cu and 1.0 mm Al filtration; dose rate factor 57 R per min; target distance 81 cm.

In experiments designed to measure proliferation of ERC, mice were given 900 R total-body irradiation with lead shielding of the right hind limb. Test animals had been given 200 R total-body irradiation with the same leg shielded 4 to 7 days prior to the larger radiation dose. One control group received only the 900 R challenge; another control group was not irradiated. On days 3 and 4 after administration of 900 R, all mice were transfused with 0.6 ml of packed red cells. Six days after the 900 R administration, all mice were given 1 unit of erythropoietin[13] subcutaneously. Forty-eight hours later, 0.5 μc $^{59}FeCl_3$ in citrate solution was given intravenously in a total volume of 0.25 ml. Three days after radioiron administration, radioiron was determined in a scintillation well counter on a 0.25 ml sample of blood from the heart. The percentage of the administered radioiron present in the peripheral blood was calculated from the activity of a dilute standard of the radioiron solution prepared at the time of administration, and assuming a blood volume of 7 per cent of the body weight.[14] Microhematocrit determinations were performed at the end of the experiment, and data from animals with hematocrits below 55 per cent were discarded because such mice were no longer sufficiently plethoric to insure suppression of endogenously stimulated erythropoiesis.

In experiments designed to measure proliferation of CFU, mice were given 200 or 250 R total-body irradiation followed immediately by 550 or 500 R administered with the left leg shielded, i.e., total body irradiation at this stage = 750 R. Test groups had received 200 R total-body irradiation with the same leg shielded 5, 8, or 13 days previously. Thus, the total whole body dose to the test groups was 950 R. Eight days after the last dose of radiation, animals were killed, the spleens were fixed in Bouin's solution, and the total number of colonies grossly visible on the surface of each spleen was counted. Spleens were supplied in a random fashion to one investigator who counted the colonies without knowing the source of the sample.

RESULTS

Effect of previous radiation damage on response to erythropoietin following a second exposure to radiation. Table 1 illustrates two separate experiments in which the recovery of response to one unit of erythropoietin after 900 R total-body irradiation with one leg shielded was determined in control plethoric animals and in plethoric mice that had received 200 R total-body irradiation with one leg shielded 5 days previously. In both experiments a substantial reduction in erythropoietic response is seen in mice that had received a massive dose of irradiation six days before administration of the challenging injection of erythropoietin. Since very few mice survive 900 R for 8 days unless the leg is shielded, and the few survivors do not respond to erythropoietin, we attribute the 4.3 per cent response to the effect of erythropoietin on the progeny of primitive cells that were in the leg at the time of irradiation, and that had been distributed between the leg and the rest of the body by the time of erythropoietic challenge. The essen-

Table 1

EFFECT OF PREVIOUS RADIATION DAMAGE ON RESPONSE TO ERYTHROPOIETIN AFTER A SECOND EXPOSURE TO RADIATION

Expt. No.	200 R, L.S. — 5 Days —	900 R, L.S. — 6 Days —	E — 2 Days —	^{59}Fe — 3 Days —	t	% ^{59}Fe ± 1 S.E.
1	-	-	X	X		9.0 ± 1.8
	-	X	X	X		4.3 ± 0.9
	X	X	X	X		11.0 + 1.6
2	-	-	X	X		13.5 + 1.6
	-	X	X	X		4.3 + 0.5
	X	X	X	X		10.4 + 1.0

L.S. = Leg shielded, E = 1 unit erythropoietin, t = time of sacrifice.

Table 2

EFFECT OF PREVIOUS RADIATION DAMAGE ON SPLEEN CLONE FORMATION AFTER A SECOND EXPOSURE TO RADIATION

200 R, L.S. — 5 Days —	500 R, L.S. 250 R, t.b. — 8 Days —	t	Spleen clones ± 1 S.E.
-	X	X	4 ± 0.7
X	X	X	10 ± 0.6

L.S. = Leg shielded, t.b. = total-body, t = time of sacrifice.

tial feature of this experiment is that mice which had been given 200 R with the leg shielded 5 days before the larger irradiation responded to erythropoietin much more vigorously than did animals not receiving the initial irradiation. Indeed, in both experiments the twice-irradiated animals showed a response that was approximately normal.

Effect of previous radiation damage on colony-forming units following a second exposure to irradiation. The initial experiment in this series measured the effect of previous irradiation on the clone-forming ability of non-plethoric mice receiving 750 R total-body irradiation with one leg protected. With complete protection of the leg, so many totipotential cells colonized the spleen that hematopoietic tissue in this organ was confluent in 8 days. After 250 R was administered to the leg, the average number of spleen colonies was reduced to 4 (15 mice). In thirteen mice which had received 200 R total-body irradiation with leg shielded 5 days before 500 R leg shielded - 250 R total-body irradiation, the average number of spleen colonies was 10. This study is summarized in Table 2.

The final experiment was designed to test whether increasing the time between first and second doses of radiation had any effect on the number of colonies appearing in the spleen. In an effort to increase the numbers of colonies in both control and test animals, the dose of radiation administered to the leg was reduced to 200 R. Table 3 shows that mice given 550 R leg shielded - 200 R total-body radiation had an average of 8 colonies per spleen after 8 days. Animals pre-treated with 200 R total-body with leg shielding 5, 8, or 13 days before being treated with 550 R leg shielded - 200 R total-body irradiation had respectively 17, 21, and 25 colonies per spleen.

Table 3

EFFECT OF PREVIOUS RADIATION DAMAGE ON SPLEEN CLONE FORMATION AFTER A SECOND EXPOSURE TO RADIATION

200 R, L.S.		550 R, L.S. 200 R, t.b.	8 Days ——— t	Spleen clones ± 1 S.E.
-	-	X	X	8 ± 2.3
X	5 days	X	X	17 ± 4.0
X	8 days	X	X	21 ± 3.4
X	13 days	X	X	25 ± 2.9

L.S. = Leg shielded, t.b. = total-body, t = time of sacrifice.

DISCUSSION

These studies were prompted by our interest in the postulation that the numbers of primitive hematopoietic cells is regulated. It is reasonable to speculate that a compartment of cells of such critical importance to an animal would have a definite mechanism for preservation of its numbers. Evidence for rapid growth of primitive hematopoietic cells is found in experiments where as few as 8×10^5 marrow cells will protect a lethally irradiated mouse.[15] Some of the transplanted primitive cells proliferate rapidly in a few days, and weeks later the marrow is completely repopulated. If this rate of proliferation were to continue, the mass of primitive

cells would reach abnormal proportions in a short time. Since equilibrium appears to be established once near-normal numbers are present, we conclude that a mechanism for regulation of cell numbers does indeed exist.

Another experimental approach, employing sublethal irradiation, has demonstrated that a primitive compartment of cells recovers from damage quickly. Following 150 R total-body irradiation, the response to erythropoietin recovers rapidly after an initial lag period of 4 days,[10] and indeed even overshoots the normal level by a substantial margin.[11] These experiments were conducted in transfusion-induced plethoric mice where no red cell formation occurs until the challenging dose of erythropoietin is administered. Hence recovery of a characteristic property of the compartment of cells, namely the response to erythropoietin, occurs even when this process is in abeyance. Again, the rapidity of recovery once regeneration begins, suggests to us a mechanism for regulation of primitive cell numbers.

If Bruce and McCulloch are correct,[12] our experiments have been directed at two different cell lines—the stem cell or colony-forming unit, and the erythropoietin-responsive cell, both of which respond similarly to the different stimuli employed. Such an observation is probably not surprising, since if the ERC is different from the CFU, the former is undoubtedly an outgrowth of, or a differentiated product of, the latter.

We interpret the results of our experiments as follows. When one leg is spared an initial radiation injury, the primitive cells within that leg are stimulated to proliferate at a rate in excess of normal. Undoubtedly many cells emerge from the protected leg and 5 days later have colonized the hematopoietic tissues elsewhere in the body. The progeny of such cells are destroyed by 900 R, but the shielding of the leg again protects the primitive cells within its marrow. In the same manner, control mice not previously irradiated receive 900 R with one leg shielded. All animals are then made polycythemic by transfusion so that the erythropoietin tolerance test[16] can be used for quantitation of the numbers of erythropoietin sensitive cells. We realize that several cell cycles elapse between the final irradiation and the administration of erythropoietin, and that the number of ERC, both those within the leg and those having migrated elsewhere, is probably substantially increased. However, we believe that response to erythropoietin on the sixth day after irradiation is related to the viable number of CFU or ERC immediately after irradiation, and that the greater response to erythropoietin in twice-irradiated animals represents enhanced proliferation of primitive cells within the leg between the first and second radiation exposure.

A similar enhancement of CFU proliferation within the leg following shielding of that leg and 200 R total-body irradiation is demonstrated by colony counts. Animals receiving 750 R total-body irradiation have no spleen clones 8 days later. If one leg is shielded, too many stem cells are protected and the clones of hematopoietic tissue are contiguous in 8 days. When, however, the protected leg is partially damaged by 200 or 250 R, only enough stem cells survive to give rise to a small number of discrete clones. Animals so damaged after having previously received 200 R total-body irradiation with leg shielding from 5 to 13 days earlier have increased numbers of clones in comparison to animals not receiving the initial irradiation. Again we interpret these findings as indicating that there are increased numbers of stem cells in the leg at the time of the second irradiation, as a consequence of the initial damage to stem cells outside the leg.

Thus, attention becomes focused on the mechanism by which the number of ERC and CFU

are regulated. Since primitive cells in one area respond when similar cells in another area are damaged, a humoral mechanism is suggested. Although factors capable of stimulating cell proliferation may arise elsewhere in the body and exert an effect on stem cells in the protected portion of the marrow, an equally plausible humoral mechanism based upon reduction of inhibition may be postulated. If it is further postulated that inhibitors are natural products of the cells whose proliferation is inhibited, it follows that a decrease in the number of these cells will temporarily suppress inhibition, through reduction of the titer of hypothetical inhibitor, until cell division has restored the population to a normal number, at which time a normal titer of inhibitor will be restored. Such a concept, first presented by Weiss,[17] is attractive because it offers a theoretical basis accounting for tissue growth without requiring a separate monitoring organ for each tissue capable of regeneration after partial ablation or destruction. The most delicately regulated system might rely upon both an inhibitor and a stimulator of cell replication, and evidence arising from other model systems for the existence of inhibitor or "retine," and promoter, or "promine," has recently been reviewed by Szent-Györgyi.[18]

The control of populations of primitive cells, and the nature of abnormal states arising from breakdown of such control, are problems of great interest. It is hoped that modifications of the model described will enable us to determine whether specific stimulators or reduction in titer of specific inhibitors account for the results here recorded.

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CHARACTERISTICS OF THE HEREDITARY ANEMIA OF Sl^m MUTANT MICE*

By

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Sl^m is one of a series of Sl-alleles of mice producing, in the homozygous condition, a macrocytic anemia associated with coat color change and sterility.[1-6] The characteristics of the different Sl-mutants are given in Table 1.

Table 1

CHARACTERISTICS OF Sl-SERIES MUTANTS

Genotype	RBC	MCV	Pigmentation	Fertility	Viability	References
Sl Sl	Greatly decreased	Greatly increased	Black-eyed white	No germ cells in neonate	Lethal	1, 2
Sl^d Sl^d	Greatly decreased	Greatly increased	Black-eyed white	Sterile	Decreased	3, 4
Sl^{so} Sl^{so}	Greatly decreased	-	Black-eyed white	Sterile	Severely decreased	5, 6
Sl^m Sl^m	Greatly decreased	Greatly increased	Black-eyed white	Sterile	Decreased	5, 6
Sl +	Decreased	Slightly increased	Dilute	Fertile	Normal	1, 2
Sl^d +	Decreased	Increased	Dilute	Fertile	Normal	3, 4
Sl^{so} +	-	-	Dilute	Fertile	Normal	5, 6
Sl^m +	Normal	Normal	Dilute	Fertile	Normal	5, 6

The triad of macrocytic anemia, coat color change, and sterility is known to occur also in W-series mutants. Although detailed studies of the anemia in Sl-series mutants have not been published, the anemia of W-series mutants has been extensively studied.[7-14] This is a pure red cell anemia associated with elevated reticulocyte counts.[7] The marrow is hypoplastic, with a predominance of early erythroid forms, suggesting a maturation arrest.[8] The anemia is gradually but completely cured by implantation of isologous fetal hematopoietic tissue from normal animals, suggesting that a defect in the marrow hematopoietic cells is responsible for the con-

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dition.[9-11] Further support for this concept comes from recent work demonstrating that the ability to form spleen-colonies is impaired in W-mutant marrow cells.[12] The anemic animals are relatively resistant to erythropoietin, although large doses produce a small but apparently significant response.[13,14] Exposure of the anemic mice to hypoxia produces an increase in the hematocrit comparable to that observed in normal mice.[7,14]

Although the syndromes of the Sl- and W-series mutants are strikingly similar, the mutations occur at different genetic loci: the Sl and W genes segregate independently with no linkage between them.[1] In addition, we have found that the anemia of Sl^mSl^m animals has several characteristics not reported in the W-series anemias. This paper describes the characteristics and mechanisms of the anemia of this mutant genotype.

METHODS AND MATERIALS

Adult C57BL/6-Sl^mSl^m, C57BL/6-Sl^m+, and C57BL/6-++ mice derived from stock originally given us by Dr. Dorothea Miller, constituted the principal experimental animal. The gene mutation in these mice was shown to be allelic to that of Sl^d mice by cross-matching with Sl^d mice supplied by Dr. E. S. Russell. Animals used in individual experiments were matched for age and sex. Six 10-week-old CBA female mice from the Jackson Laboratory were used for erythropoietin assay. Retired CF No. 1 breeder females were used as red cell donors in producing polycythemia.

Erythropoietin was obtained from the United States Public Health Service, Hematology Section.

Red cell and white cell counts were determined in the standard manner on blood from a tail vein. Hematocrits were measured by the microhematocrit method, and hemoglobin levels by the cyanmethemoglobin method. The reticulocyte percentage was determined by counting 1,000 cells on a smear prepared with brilliant cresyl blue stain. Platelets were counted by the modified direct method of Skirmont et al.[15]

Gastrointestinal blood loss was estimated in the following manner. C57BL/6-++ mice were injected intraperitoneally with 1.5 μc $^{59}FeCl$* and exsanguinated 3 days later to obtain ^{59}Fe labeled red cells. Two-tenths ml of a 50 per cent suspension of labeled red cells in saline was injected intravenously into each animal to be tested. The mice were kept in individual metabolism cages and the stools collected daily for 2 weeks. The radioactivity of the stools was determined in a well type scintillation counter. The specific activity of the blood was measured on the first and last days. The specific activity of the blood on intermediate days was estimated by interpolation on semi-log paper. Daily blood loss in ml was then calculated as the dividend

$$\frac{\text{total activity of the stools on day x}}{\text{specific activity of the blood on day x}}.$$

^{59}Fe incorporation into the circulating red cells, as a test of erythropoietic response or as an erythropoietin assay, was determined by the method of DeGowin et al.[16] Mice were injected subcutaneously with erythropoietin or plasma no sooner than 2 days after being made polycythemic, and 58 hours later 0.5 μc $^{59}FeCl$ in 0.2 ml saline was injected intravenously. Seventy-two hours later a measured amount of blood was obtained by cardiac puncture or from a tail vein and counted in a well type scintillation counter. The percentage of ^{59}Fe incorporation was cal-

*Specific activity 36 mc per mg Fe.

culated on the basis of an estimated blood volume in plethoric mice of 7 per cent of body weight.

Mice were made polycythemic either by intravenous injection of 0.5 ml of an 80 per cent suspension of washed red cells in saline, or by intraperitoneal injection of 1.0 ml of such a suspension on 2 consecutive days. Booster transfusions were given as necessary to maintain the hematocrits between 55 and 65 per cent. Animals with hematocrits outside these limits were excluded from the experiment.

Hypoxia was produced in the hypobaric chamber described by DeGowin et al.[17]

RESULTS

The peripheral blood parameters of adult Sl^mSl^m (mutant homozygote), Sl^m+ (mutant heterozygote) and ++ (normal) mice are recorded in Table 2. Sl^m+ mice and ++ mice differ little in these parameters. In contrast, the Sl^mSl^m animals have a macrocytic anemia with an elevated reticulocyte count. The reduction in cell number is limited to the erythrocytes, there being no depression of the leukocyte or platelet count. The bone marrows are normocellular with a normal ratio of myeloid to erythroid cells.

Table 2

PERIPHERAL BLOOD PARAMETERS OF NORMAL AND Sl^m-MUTANT MICE. VALUES REPRESENT THE AVERAGE OF 10 ANIMALS ± 1 S.D.

	++	Sl^m +	Sl^m Sl^m
Hgb (Gm %)	15.4 ± 1.0	15.2 ± 0.7	11.5 ± 1.9
Hct (%)	50 ± 1	48 ± 3	39 ± 5
RBC x $10^6/mm^3$	8.16 ± 0.88	7.89 ± 0.66	5.42 ± 1.06
MCV (μ^3)	61 ± 4	61 ± 4	73 ± 7
Retic (%)	3.6 ± 1.0	2.5 ± 1.7	7.6 ± 4.8
WBC/mm^3	11,000 ± 3,000	15,000 ± 7,000	20,000 ± 9,000
Platelets x $10^6/mm^3$	1.5 ± 0.2	-	1.5 ± 0.3

The elevation of the reticulocyte count suggested that either hemolysis or bleeding might be the basis of the anemia. Occult blood was demonstrable by the benzidine method in stools of the anemic animals, where a strikingly more prompt and intense response was noted than in control mice, and it therefore seemed important to compare quantitatively the amount of gastrointestinal blood loss in normal and mutant mice. As shown in Figure 1, Sl^m+ and ++ mice lost radioiron equivalent to 3.1 to 3.3 microliters of blood daily. In contrast, Sl^mSl^m mice lost radioiron equivalent to 34 microliters daily, over 10 times as much as the normals. Since the test animals averaged 20 g in total body weight, this amount approximated 3.5 per cent total blood volume daily, assuming a blood volume of 5 per cent of body weight.

In order to test whether blood loss of this magnitude could entirely account for the anemia, we subjected normal mice to blood loss approximating that observed in Sl^mSl^m mice. An amount of blood equal to 3.5 per cent of the estimated blood volume (again assuming a blood volume of 5 per cent of body weight) was taken from a tail vein every day. Within 20 days the blood parameters had stabilized at new steady-state values. These values are compared to those of the ane-

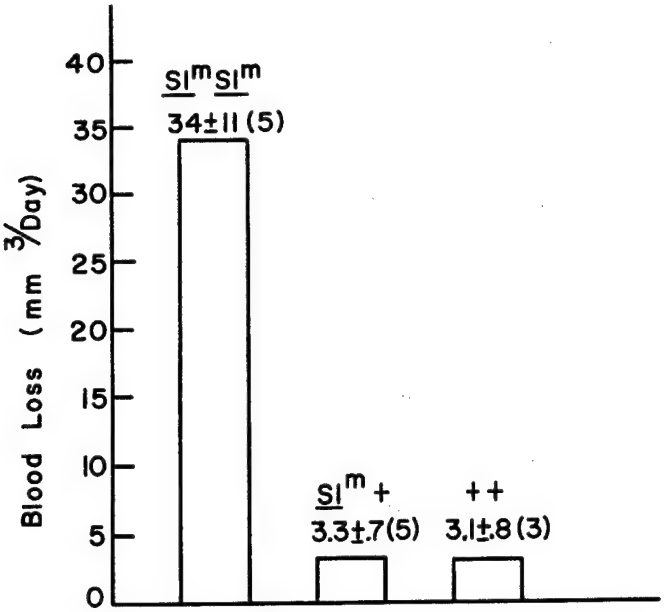

Figure 1. Average ± 1 S.D. ( ) = number of test animals.

mic mice in Figure 2. The normal mice respond to what is presumed to be comparable blood loss with a greater reticulocytosis and do not become as anemic as the mutant animals.

Failure of normal erythropoietic response to bleeding might result from a failure to elaborate sufficient quantities of erythropoietin, from rapid inactivation of erythropoietin, or from failure of the marrow to respond normally to the erythropoietin produced. In order to determine if there was a reduction in the rate of production or an increase in turnover of erythropoietin, we measured the plasma erythropoietin levels of the anemic animals. As shown in Figure 3, Sl^mSl^m plasma produces a striking erythropoietic response in normal mice, demonstrating that even under normal conditions the plasma of Sl^mSl^m mice is characterized by high concentrations of erythropoietin.

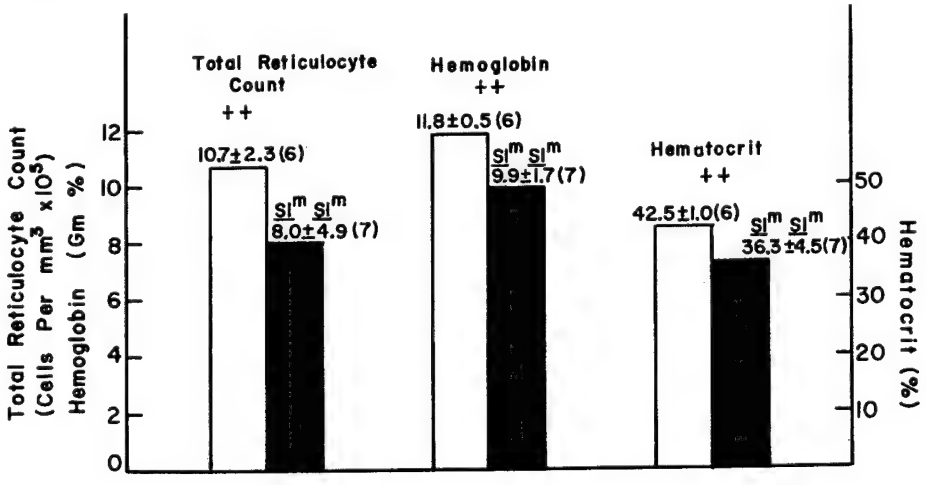

Figure 2. Average ± 1 S.D. ( ) = number of test animals.

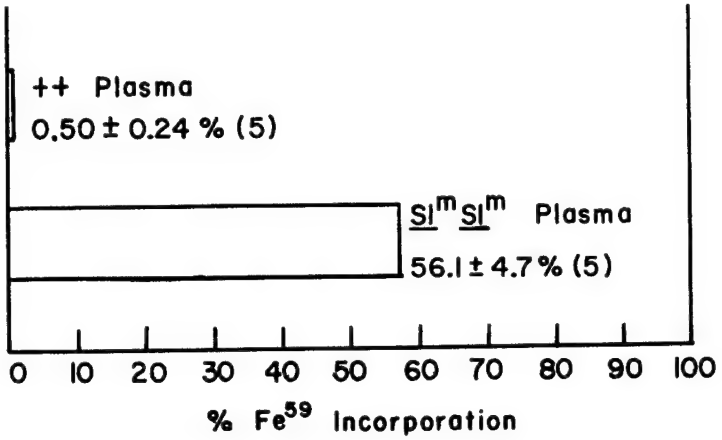

Figure 3. Average ± 1 S.D. ( ) = number of assay animals. Volume of plasma assayed = 0.5 ml.

Failure of normal response to erythropoietin might result if after prolonged bleeding the mutant animals developed iron deficiency of sufficient magnitude to depress erythropoiesis. We therefore administered iron to the anemic animals in an attempt to ameliorate the anemia. One mg of iron, in the form of a saccharated iron oxide preparation* was injected intraperitoneally on day 0 and an additional 2 mg on day 23. Hemoglobin levels and reticulocyte counts were measured at intervals, as indicated in Figure 4. The anemic animals failed to respond to iron with elevations either of reticulocyte count or hemoglobin level.

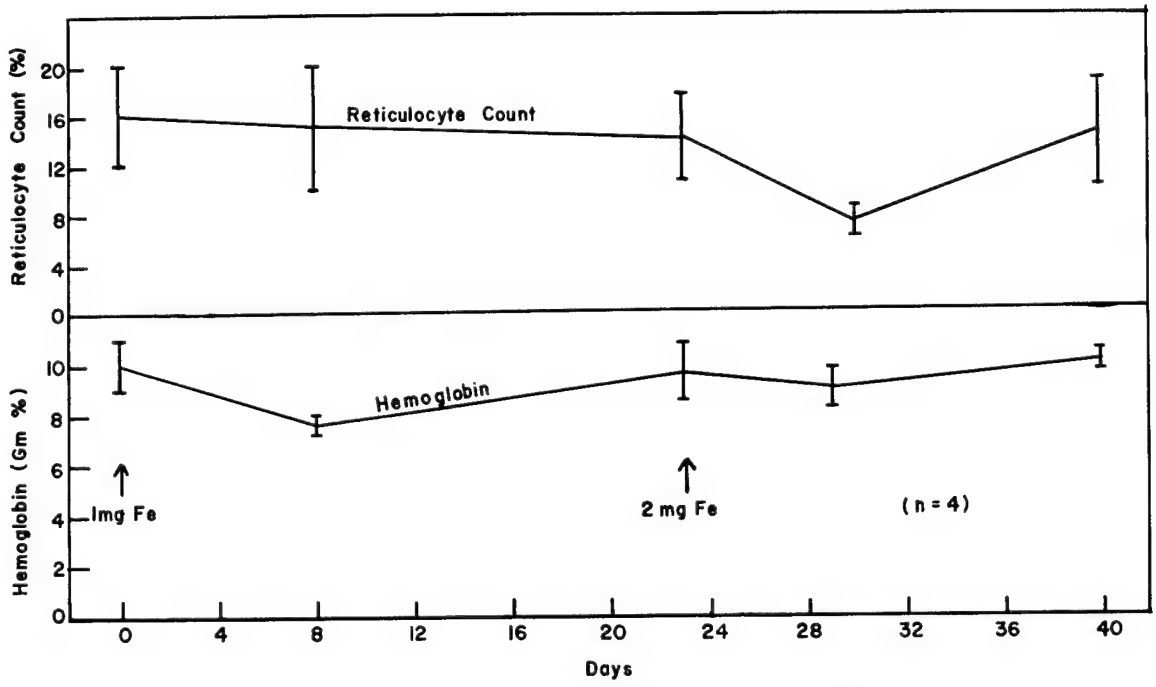

Figure 4. The brackets indicate the standard error of the mean. n = number of test animals.

*Proferrin, produced commercially by Merck, Sharpe and Dohme.

We next tested the response of the mutant animals to exogenous erythropoietin. Erythropoietin was administered to plethoric test animals and the resulting ^{59}Fe incorporation into red cells was determined. The results are shown in Figure 5. In Experiment A, plethoric ++ and Sl^m+ mice were given 3 injections of 0.33 units of erythropoietin at 5 hour intervals. The normal mice responded with ^{59}Fe incorporation several times that of the mutant heterozygotes. In Experiment B, a single injection of 3 units of erythropoietin produced a markedly greater erythropoietic response in normal mice than in the mutant homozygotes. The homozygotes showed no greater ^{59}Fe incorporation after this dose of erythropoietin than did normal controls given no erythropoietin.

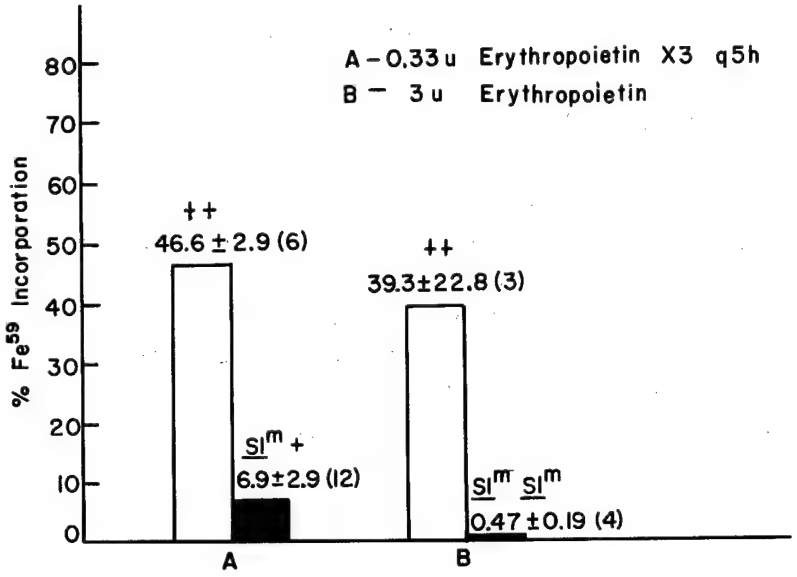

Figure 5. Average ± 1 S.D. ( ) = number of test animals.

To determine if the homozygotes were capable of responding to larger doses of erythropoietin, plethoric homozygotes were given 20 units of erythropoietin on 4 successive days. This elicited a reticulocytosis of 1.2 per cent on the fourth day after the last injection (Figure 6).

To further characterize the erythropoietic defect in the mutant mice, we tested the ability of Sl^mSl^m mice to respond to a hypoxic stimulus. Sl^mSl^m and ++ mice were made comparably polycythemic by hypertransfusion and placed in a hypoxic chamber at 0.5 atm. Serial reticulocyte counts and plasma erythropoietin determinations were used to follow the response to hypoxia. The results are shown in Figure 7. As compared to normal animals, Sl^mSl^m mice were markedly deficient in their reticulocyte response, despite the production of high plasma levels of erythropoietin.

In a companion experiment, we compared the responses of Sl^m+ and ++ mice to hypoxia. Since Sl^m+ mice are not anemic, it was not necessary to transfuse the animals in order to assure comparable hemoglobin levels. The animals were placed in the hypoxic chamber at 0.5 atm and their response followed by reticulocyte counts and hemoglobin determinations. The results are shown in Figure 8. After 4 days of hypoxia, normal mice exhibited a significantly greater rise ($p < 0.05$) in reticulocyte count than did the heterozygotes. By 10 days, however, this dif-

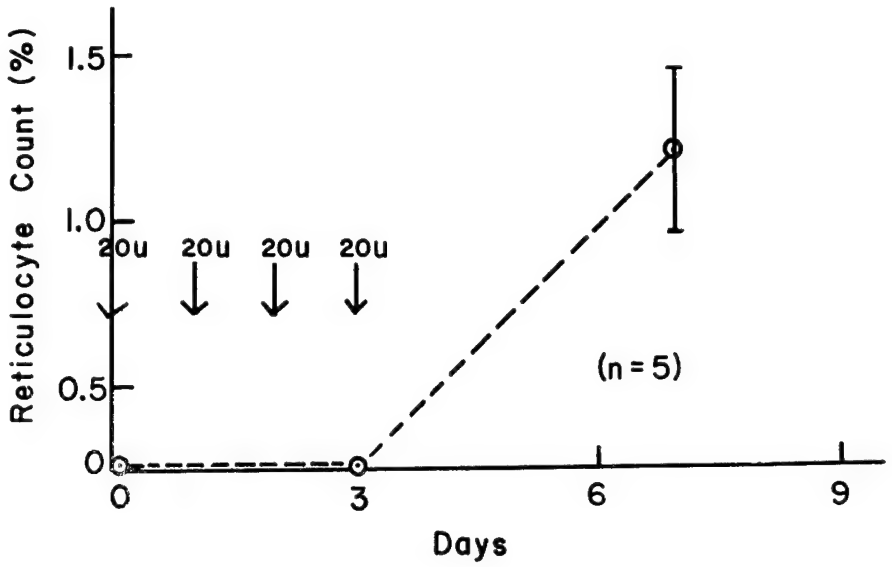

Figure 6. The brackets indicate the standard error of the mean. n = number of test animals.

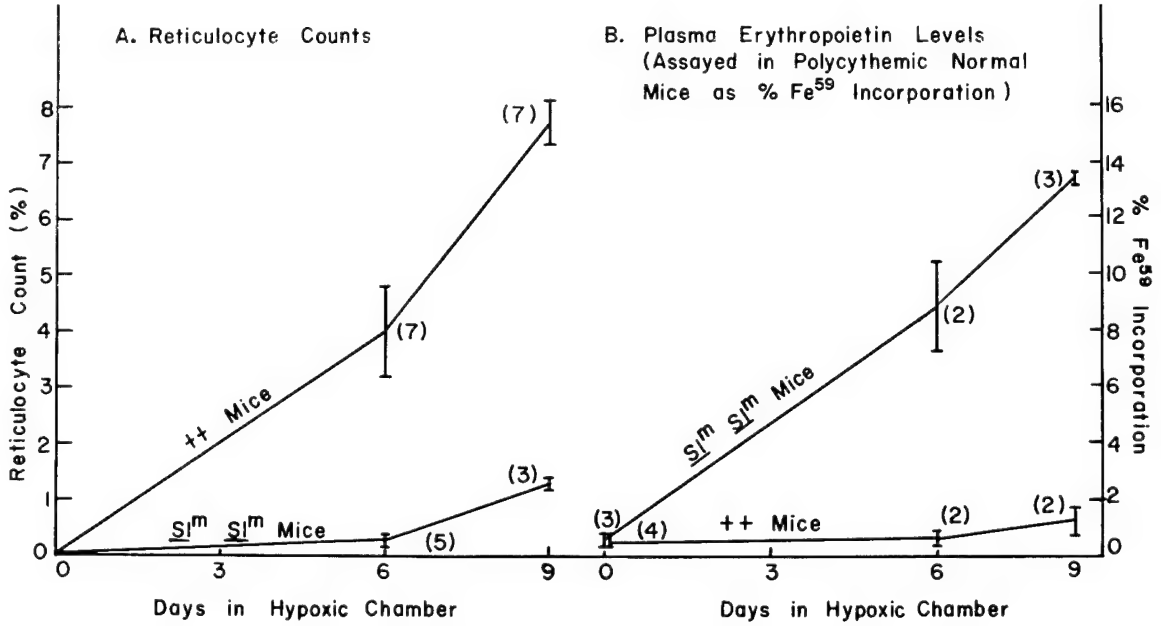

Figure 7. The brackets indicate the standard error of the mean. ( ) = number of test animals. Volume of plasma assayed = 0.5 ml.

ference had disappeared. The rise in hemoglobin levels during the 10 days was not significantly greater ($p < 0.05$) in the normals than in the heterozygotes.

DISCUSSION

These studies suggest that two etiologic factors, gastrointestinal bleeding and erythropoietin defect, underlie the anemia of Sl^mSl^m mice. The existence of gastrointestinal bleeding is postulated on the basis of evidence indicating that large amounts of radioactivity appear in the

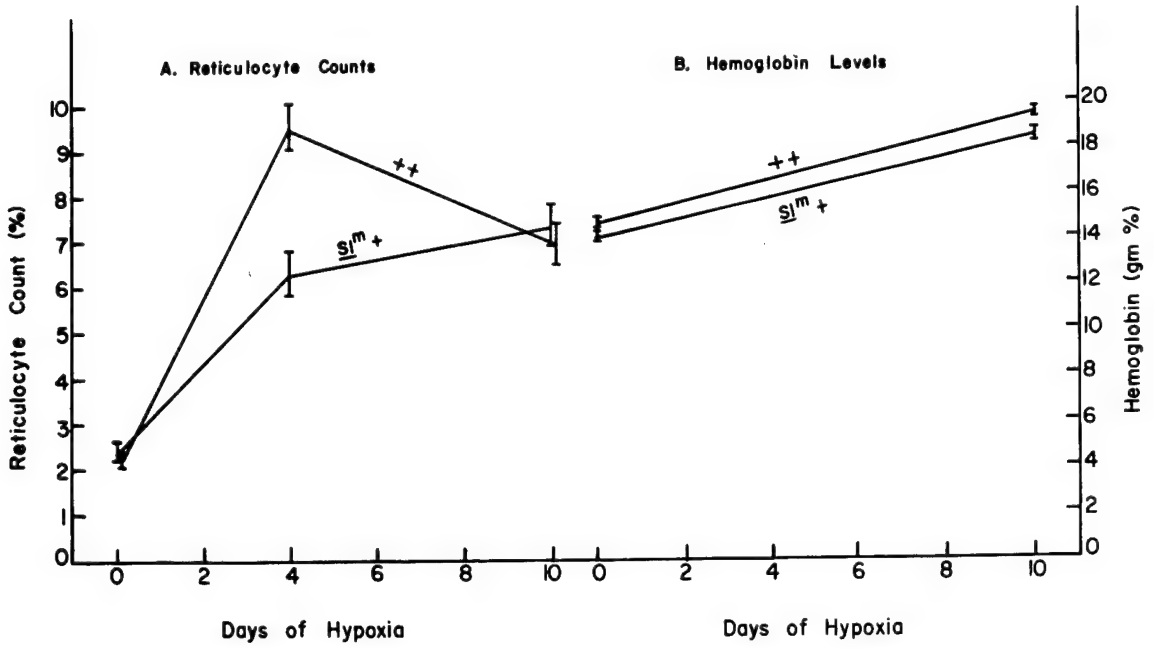

Figure 8. The brackets indicate the standard error of the mean. Number of test animals = 16.

stools after transfusion of ^{59}Fe labeled red cells into the anemic animals. It should be pointed out that we have actually demonstrated only that ^{59}Fe is lost into the gastrointestinal tract, not that red cells are. However, if there is no bleeding, then the appearance of ^{59}Fe in the stools must be explained by the coincidence of two defects, one leading to removal of ^{59}Fe from the red cells and the other to transport of ^{59}Fe into the intestinal lumen. We would also need to hypothesize a defect leading to transport of ^{51}Cr into the intestinal lumen, for when ^{51}Cr labeled red cells are employed, the results obtained are similar to those with ^{59}Fe labeled red cells.[20] We therefore favor the simpler hypothesis that a gastrointestinal bleeding defect exists.

We have been unable to find a cause for this bleeding. The gastrointestinal tract appears grossly normal and several random sections of the stomach and intestine failed to show erosion or ulceration. A detailed histological study of the entire gastrointestinal tract remains to be undertaken. There is no evidence of bleeding in sites other than the gastrointestinal tract, and the platelet count is normal.

Despite their continued bleeding, iron deficiency will not completely explain the impaired erythropoiesis of Sl^mSl^m mice, since administration of iron does not ameliorate their anemia.

Gastrointestinal bleeding has not been reported in W-series mutants. Our demonstration of an erythropoietic defect in Sl^m-mutants, however, shows that their similarity to W-series animals is more than superficial. The erythropoietic defect renders Sl^m-mutants, the heterozygote as well as the homozygote, relatively refractory to erythropoietin. We have, in fact, been unable to demonstrate a significant response to a single injection of erythropoietin in the homozygotes, although repeated high doses do induce a clear-cut reticulocytosis in plethoric homozygotes. This relative insensitivity to erythropoietin is also a characteristic of W-series mutants.[13,14]

Sl^m-mutants are defective in their response to hypoxia as well as to erythropoietin. In the homozygotes there is markedly less erythropoietic response to hypoxia than in normal animals

despite the production of high plasma concentrations of erythropoietin. In the heterozygotes the defect is much less apparent, and is evidenced only by a lag in achieving a reticulocyte peak. Thus, although the normal mouse shows a greater reticulocytosis than the heterozygote after 4 days of hypoxia, by 10 days the difference in reticulocyte count no longer exists.

Keighley et al.[13] have reported normal response to hypoxia in WW^v mice, despite a markedly decreased sensitivity to erythropoietin. Although the apparently normal response of WW^v mice to hypoxia, as contrasted to the abnormal response of Sl^mSl^m mice, may represent another difference between these mutants, it may also be attributable to differences in experimental conditions. The experiments with WW^v mice were conducted with anemic animals, and the effect of hypoxia on these animals was compared to its effect on mice with normal hematocrits. It is possible that an already anemic animal would be sufficiently sensitive to hypoxia to respond with production of enough erythropoietin to stimulate even a defective marrow to a high rate of erythropoiesis. Our experiments were conducted in plethoric animals where comparable hematocrits excluded the possibility that different cell volumes might contribute to the results observed.

In W-series mutants, it has been established that the observed erythropoietic defect is an intrinsic cellular one, since implantation of hematopoietic tissue from normal animals abolishes the anemia.[9-11] Experiments to determine if this is also true for Sl^m-mutant animals are in progress.

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THE EFFECT OF ERYTHROPOIETIN ON HUMAN BONE MARROW CELLS IN VITRO*

By

S. B. Krantz†

We have previously reported on a cell culture system for rat bone marrow which responds to the hormone, erythropoietin, with a marked increase in hemoglobin production.[1] Further study of this system has revealed that the hormone acts within 15 minutes to increase ^{14}C-uridine incorporation into an RNA that has many of the properties of messenger RNA.[2] The method thus provides a useful tool for studying the mechanism of action of erythropoietin. The experiments described here show that human bone marrow can also be used in this system and that, with a few modifications, a response to erythropoietin equivalent to that observed with rat marrow, can be obtained. Thus the system is usable for the study of those human red cell diseases in which there is an unexplained deviation from normal red cell production.

MATERIALS AND METHODS

Bone marrow was aspirated by sternal puncture and suspended as previously described[1] in 3 ml NCTC-109, with 50 units of penicillin and 170 units of heparin per ml. The cells were washed once in sterile centrifuge tubes with 3 ml of the same solution, resuspended, and 0.48 ml pipetted into 35 x 10 mm sterile tissue culture dishes (Falcon Plastics). Thirty-two hundredths milliliter of heparinized homologous plasma from a normal donor of AB blood type was then added. In some experiments, 0.16 ml of this plasma was replaced by sterile-filtered, precolostrum, newborn calf serum (Colorado Serum Company). The rest of the procedure was unmodified from the previous report except for the substitution of cyclohexanone for butanone-2.‡ ^{59}Fe was added as indicated in the figures, and the heme solution was dried on aluminum planchets and counted in a gas-flow geiger counter. The sheep plasma erythropoietin (Step IV, lot K 103214A) was obtained from the United States Public Health Service Study Section on Hematology.

RESULTS AND DISCUSSION

Initially, the medium used consisted of 60 per cent NCTC-109 with 40 per cent homologous plasma since this seemed important to the rat marrow system.[3] In order to circumvent the blood group antigen-antibody problem and to provide a common pool of plasma for all human marrows, plasma from AB donors was used since this does not have antibody to the major blood groups. A dose response curve with erythropoietin in this system is shown in Figure 1 and demonstrates a maximum 3-fold stimulation of heme synthesis above the control. Since the stimulation of heme synthesis by erythropoietin can be increased in the rat system with the use of fetal

*This report is taken from a paper that appeared in Life Sciences, 4:2393, 1965.

†Leukemia Society Scholar.

‡E. Goldwasser, unpublished work.

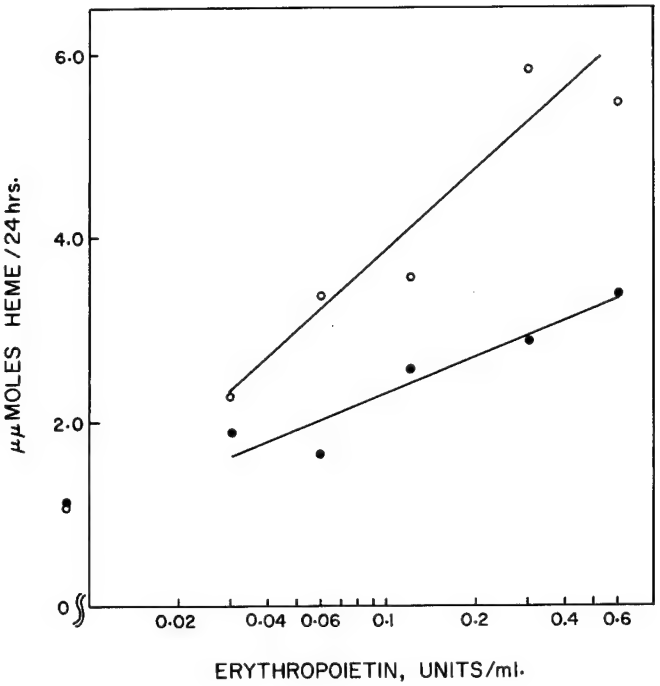

Figure 1. Erythropoietin dose response curve for human marrow cells in vitro. The marrow was obtained from a normal 25-year-old male. Four hundred eighty nucleated cells per mm^3 and 100,800 red blood cells per mm^3 were present in each plate. Fifty-five hundredths microcurie ^{59}Fe in 0.1 ml medium was added after 46 hours of incubation, and cultures were terminated 24 hours later. (•) 40% homologous plasma, (○) 20% homologous plasma and 20% newborn calf serum. Each point is the mean of triplicates.

calf serum[3] or newborn calf serum,* half of the homologous plasma was replaced with the latter. As demonstrated in Figure 1 the dose response curve with human marrow then shows a 6-fold increase of heme synthesis above the controls. This is equivalent to the response observed with marrow from non-fasting rats.[1] Fasting of human subjects would presumably increase the response even more if this were desired, but would add an additional burden and is not absolutely necessary for adequate interpretation of this experimental system. Figure 1 also demonstrates that human marrow responds to the same range of concentrations of the hormone as rat marrow.

The change in rate of heme synthesis by human marrow cells with time of incubation, and the effect of erythropoietin on this change are shown in Figure 2. The decline in rate of heme synthesis without erythropoietin is similar to the decline previously seen with rat marrow.[1] The addition of the hormone to human marrow, however, provokes a slower and more prolonged increase in rate of heme synthesis, above the controls, than is observed with rat marrow. Sixty-six hours after beginning the cultures the erythropoietin treated marrow still maintains a rate of heme synthesis about 90 per cent of the initial rate. While this may simply be related to the longer maturation time of human marrow cells compared to rat cells, it also suggests that human marrow may be a better tissue for long term maintenance in vitro.

*E. Goldwasser, unpublished work.

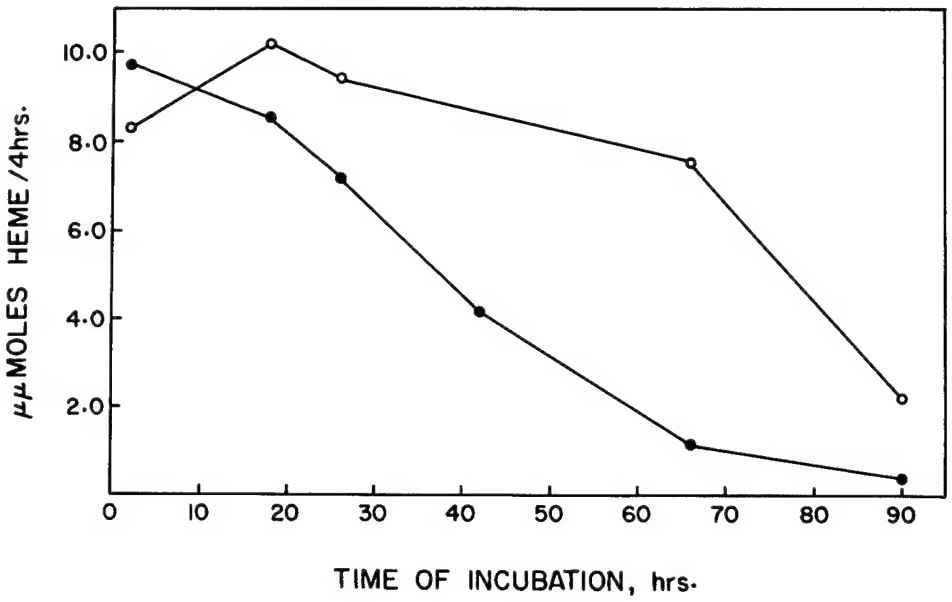

Figure 2. Effect of erythropoietin with time of incubation on human marrow cells in vitro. The marrow was obtained from a 76-year-old female (hematocrit 30%). Three thousand nucleated cells per mm^3 and 270,000 red blood cells per mm^3 were present in each plate with 20% homologous plasma and 20% newborn calf serum. Controls (•) had no added erythropoietin; stimulated (○) had 0.3 units per ml. Each point is the mean of duplicates and indicates the middle of a 4-hour incubation period with 0.95 μc ^{59}Fe per ml.

Other reports of the effect of erythropoietin on heme synthesis by human marrow cells in vitro have been previously published,[4,5] but the effect observed was very small. The cell culture system for human marrow which is described here provides a large and unequivocal response to the hormone which allows easy and precise interpretation of experiments and should prove useful in the study of human red blood cell diseases.

ACKNOWLEDGMENTS

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THE EFFECT OF IONIC STRENGTH UPON THE HEMOLYSIS OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA ERYTHROCYTES*

By

S. Yachnin†

The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNHE) displays the same cation requirements and pH optimum as classical immune hemolysis when human serum is used as the source of complement (C').[1,2] A variety of activators of the first complement component (C'1), or C'1 esterase per se, can enhance PNHE lysis. This phenomenon suggests that PNHE hemolysis differed from classical immune sensitized sheep red cell (EA) hemolysis in that the early steps of C' activation occur in the fluid phase, leading to direct attack of PNHE by late acting complement components.[3] More recent observations made in this laboratory have confirmed this speculation; C'3a isolated by TEAE chromatography is able to attach directly to PNHE, and the resultant intermediate complex, $PNHEC'_{3a}$, resembles in all respects its counterpart in immune hemolysis, $EAC'_{1,4,2,3a}$. Both intermediates lyse in high dilutions of human serum in the absence of Ca^{++} or Mg^{++}, and both are lysed by a mixture of C'3b and C'3c isolated by DEAE cellulose chromatography. These observations led to the conclusion that PNHE hemolysis in acidified human serum in vitro is mediated by the C' system.[4]

The role of ionic strength in controlling the efficacy of classical immune hemolysis by C' has been the subject of several recent reports.[5-8] These studies have demonstrated that reduction in ionic strength results in substantial increase in C' titer. The present report concerns the effect of reduced ionic strength on PNHE lysis. The results demonstrate that PNHE lysis, like C' dependent immune hemolysis, is enhanced by low ionic strength.

MATERIALS AND METHODS

The following have all been described previously: The preparation of barbital buffered saline (BBS) at various pH's;[2] the collection and storage of human serum;[3] the collection and storage of normal human red cells and PNHE;[3] the estimation of acid hemolysis in vitro with or without various additions to the hemolytic system;[3] the isolation of C'1 esterase;[3] the isolation of C'3a;[4] the preparation and lysis of $PNHEC'_{3a}$.[4] Isotonic buffers of varying ionic strength were prepared using sucrose-BBS or mannitol-BBS as described by Rapp and Borsos.[8]

Human serum at various ionic strengths was prepared as follows: a series of buffers at μ = 0.150, 0.122, 0.094, 0.065, 0.037, and 0.009 was prepared using proportionate amounts of BBS and either mannitol-BBS or sucrose-BBS, pH 6.5. Ten ml serum was dialyzed for 16 hours at 4°C, against 500 ml of each buffer. Following dialysis, precipitate formation increased in quantity as ionic strength decreased, starting at μ = 0.094. Preliminary tests showed that the results were not altered if hemolysis was performed in the presence or absence of the precipitate, therefore it was usually removed by centrifugation before the hemolytic experiment. The

*This paper appeared in Proc. Soc. Exptl. Biol. Med., 120;715, 1965. The work was supported in part by the Joseph and Helen Regenstein Foundation.

†John and Mary R. Markle Scholar in Academic Medicine.

sera were then all readjusted to pH 6.5 with one or two drops of 0.3 N HCl and were left on ice until used.

In studying the effects of ionic strength on the hemolysis of PNHE, 0.05 ml of a 20 per cent PNHE suspension in 0.15 M NaCl was added to 1 ml of serum. In the experiments utilizing polyinosinic acid (poly I) or C'1 esterase the cells and serum were mixed in similar volumes, and 0.1 ml of the material in question dissolved in 0.15 M NaCl was immediately added. All hemolytic tests using PNHEC'$_{3a}$ were performed in serum containing 0.0075 M Na_3HEDTA. All ionic strengths have been corrected for contributions made by the various additions; the ionic strength of 0.0075 M Na_3HEDTA was taken as 0.045.

RESULTS

Starting at μ = 0.096 there is a gradual rise in PNHE lysis as ionic strength decreases. Hemolysis of PNHE reaches a maximum at μ = 0.042 and diminishes as the ionic strength is then lowered to 0.016. PNHE lysis varies somewhat with the nature of nonelectrolyte used to maintain tonicity; however, in contrast to immune lysis,[8] sucrose provides a more favorable milieu than mannitol. Normal red cells tested under similar conditions show no hemolysis. Heating the serum at 56° for 30 minutes destroys its capacity to hemolyze PNHE at all of the ionic strengths tested (Table 1, Figure 1).

Both poly I and C'1 esterase enhance PNHE lysis, and in both cases the enhancement of hemolysis observed at μ = 0.150 is greater than that produced by simply lowering the ionic strength. At ionic strengths below μ = 0.150 the addition of these enhancing materials does not further increase hemolysis beyond the values observed when they are used at μ = 0.150; i.e., the enhancing effects of a C'1 activator or C'1 esterase and decreased ionic strength are not additive (Table 2).

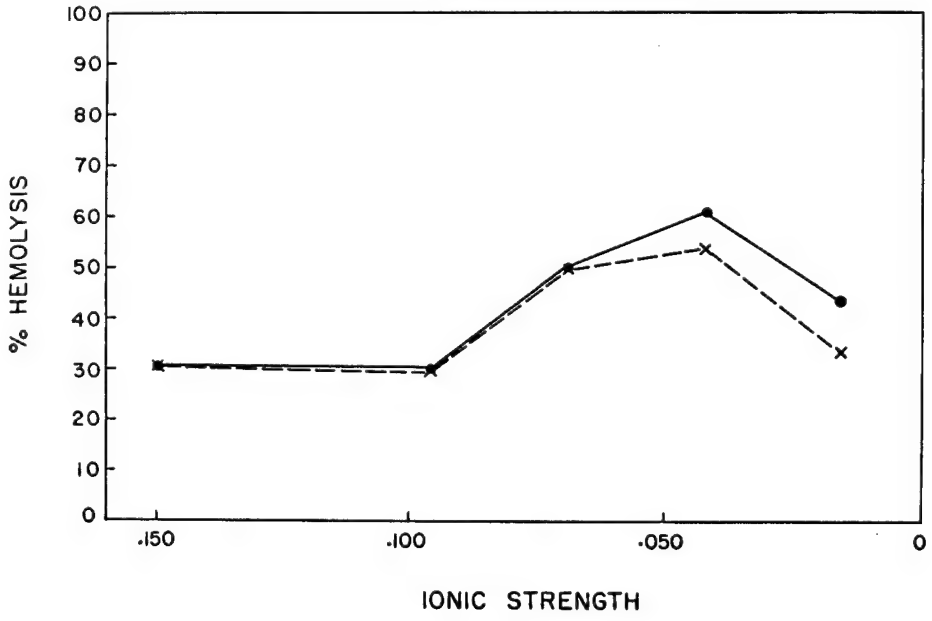

Figure 1. Effect of ionic strength upon PNH red cell hemolysis.
●———● Sucrose-BBS x - - - - x Mannitol-BBS

Table 1

	20% Suspension PNHE (ml)	20% Suspension normal red cells (ml)	Serum ionic strength* 0.150 (ml)	0.123† (ml)	0.096† (ml)	0.069† (ml)	0.042† (ml)	O.D. 540 mμ	% Lysis
A.									
PNH red cells + serum	0.05		1.0					0.910	45.9
	0.05			1.0				0.915	46.2
	0.05				1.0			1.004	50.8
	0.05					1.0		1.625	82.1
	0.05						1.0	1.785	90.2
	0.05		0.150					1.980	100% (F and T)‡
B.									
PNH red cells + heated	0.05		1.0					0.010	0.5
serum (56°, 30 minutes)	0.05			1.0				0.048	2.4
	0.05				1.0			0.060	3.0
	0.05					1.0		0.080	4.0
	0.05						1.0	0.075	3.8
C.									
Normal red cells +		0.05	1.0					0.012	0.4
serum		0.05		1.0				0.038	1.3
		0.05			1.0			0.023	0.8
		0.05				1.0		0.025	0.9
		0.05					1.0	0.036	1.2
		0.05	1.0					2.95	100% (F and T)‡

*In this and all subsequent graphs and tables, the ionic strength shown is the final one obtaining after all additions have been made.

†Dialyzed versus Mannitol-BBS.

‡Freeze and thaw.

Table 2

ABILITY OF POLYINOSINIC ACID AND C'1 ESTERASE TO ENHANCE PNHE LYSIS AT VARYING IONIC STRENGTHS

Serum ionic strength	Test material					
	Saline control		Poly I (7 μmol/ml)		C'1 esterase (60 units/ml)	
	O.D. 540	% Lysis	O.D. 540	% Lysis	O.D. 540	% Lysis
0.150	0.300	14.8	1.030	50.7	0.632	31.1
0.126	0.294	14.5	1.100	54.2	0.635	31.3
0.102			1.060	52.3	0.663	32.6
0.076	0.446	22	1.060	52.3	0.649	31.9
0.052	0.555	27.3	1.063	52.4	0.690	34

The hemolytic system consisted of 1 ml of serum, pH 6.5, at varying ionic strengths, 0.05 ml of a 20 per cent suspension of PNHE in 0.15 M saline, and 0.1 ml of 0.15 M NaCl or test material, added in the order described. The degree of hemolysis was determined after 30 minutes incubation at 37°. The ionic strengths shown are the final ones calculated after all additions to the hemolytic system had been made. One hundred per cent O.D. 540 mμ = 2.03 (freeze and thaw).

Low ionic strength has little or no effect on the ability of isolated C'3a to combine with PNHE (Table 3). The hemolysis of the intermediate complex PNHEC'$_{3a}$ in EDTA serum is inhibited by decreasing ionic strength (Table 4).

Table 3

EFFECT OF IONIC STRENGTH ON THE FORMATION OF PNHEC'$_{3a}$

PNHEC'$_{3a}$ formed at ionic strength	O.D. 540 mμ	% Lysis
0.150	1.180	46.8
0.037	1.140	45.2
100% lysis (freeze and thaw)	2.524	

Equal buttons of PNHE washed 3 times in 0.15 M NaCl, and once in BBS (μ = 0.150) or mannitol-BBS (μ = 0.037) were exposed to 0.2 ml C'3a (500 μg protein/ml) in the indicated buffer for 5 minutes at 37°. They were then washed 2 times at 0° C in the same buffer and suspended in 1 ml serum, pH 6.5, containing 0.015 M Na_3HEDTA; hemolysis was performed at 37° for 30 minutes. PNHE will not hemolyze under these conditions.

DISCUSSION

Recent work in this laboratory has emphasized the role of the C' system in PNHE in vitro acid hemolysis.[1-4] The present observations demonstrate another similarity between the PNHE hemolytic system and classical C' dependent immune hemolysis; both are enhanced by reducing ionic strength. Lysis of sensitized erythrocytes proceeds best at μ = 0.055. The optimum ionic strength for PNHE lysis is 0.042.

Table 4

EFFECT OF IONIC STRENGTH ON THE HEMOLYSIS OF PNHEC'$_{3a}$ BY EDTA-SERUM

EDTA-serum ionic strength	PNHE		PNHEC'$_{3a}$	
	O.D. 540 mμ	% Lysis	O.D. 540 mμ	% Lysis
0.195	0.011	0.6	0.477	30
0.082	0	0	0.152	9.5
0.054	0.017	0.9	0.085	5.3
100% lysis (freeze and thaw)	1.816		1.598	

The hemolytic system consisted of 1.05 ml serum, pH 6.5, at varying ionic strength containing 0.0075 M Na_3HEDTA and 0.05 ml of a 20 per cent suspension of the appropriate cell type. Hemolysis was carried out at 37° for 30 minutes. The ionic strengths shown include that contributed by the EDTA (μ 0.0075 M EDTA = 0.045).

Reduction of ionic strength increases the efficiency of immune hemolysis by several mechanisms. Included amongst these are (1) more efficient binding of C'1a to EA with resultant increase in the efficiency of EAC'$_{1,4}$ and EAC'$_{1,4,2}$ formation,[8] and (2) an enhancement of the reaction EAC'$_{1,4,2}$ + C'3 → hemolysis.[5,7,8] The latter effect has been shown to be paradoxically dependent on the number of C'2 sites per cell.[8] The precise intermediate steps favored by low ionic strength in the reaction between EAC'$_{1,4,2}$ and the C'3 subcomponents are unknown. The present data suggest that low ionic strength favors reactions involved in attaching C'3a to the cell, rather than later steps in immune lysis. Assuming that the C'3a capable of direct attachment to PNHE is already in an activated form, and recognizing that its attachment to PNHE is not enhanced by low ionic strength, the suggestion is made that in immune hemolysis low ionic strength favors the activation of C'3a by C'2 at the cell membrane. PNHE hemolysis, dependent upon fluid phase processes of C'3a activation, is enhanced by a similar mechanism involving fluid phase C' components. In addition, reduction in ionic strength may also serve to initiate a sequence of events involving early C' components similar to those initiated by C'1 esterase or C'1 activators, since the observation that the stimulation of PNHE lysis by reducing ionic strength and the stimulation of PNHE lysis by C'1 esterase or poly I are not additive, suggests that they operate via some mechanism common to both.

ACKNOWLEDGMENT

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IMMUNOLOGIC COMPETENCE OF THYMECTOMIZED RATS TO SEVERAL SOLUBLE AND PARTICULATE ANTIGENS*

By

J. L. Pinnas and F. W. Fitch

The role of the thymus in immunity has attracted substantial interest following publication of reports that neonatal thymectomy suppresses the immune response.[1-4] Several studies have indicated, however, that the antibody response of the rat is not inhibited for all antigens after neonatal thymectomy.[5-7]

This study was undertaken in search of an explanation for the difference in antibody responses to different antigens in thymectomized rats. Some rats received two different antigens, given sequentially, to determine whether the thymectomized rat with an impaired response to one antigen was capable of responding normally to a second antigen. Several soluble and particulate antigens were injected at different ages after neonatal thymectomy to evaluate both the effects of the physical state of the antigen and the age of the animals upon the antibody response. The results suggest that immunological maturity is not reached simultaneously for all antigens. Neonatal thymectomy apparently impairs antibody formation to a greater extent in systems that reach immunological maturity at an older age.

Animals and Surgical Technique

Wistar-derived CFN rats† were thymectomized in the first 24 hours of life with hypothermia as anesthesia. The thymus was removed through a mid-sternal incision using blunt dissection and suction. The operative mortality was about 10 per cent. In other rats from the same litters, a sham operation was performed in which the thymus was exposed in a similar manner, but not removed. Several rats were left intact to serve as non-operated controls. Since non-operated and sham-operated rats responded to antigen similarly they are designated simply as non-thymectomized. The runting syndrome developed in about 20 per cent of the thymectomized animals. Neither these animals, nor other animals with a poor early weight gain are included in the data.

Preparation and Administration of Antigens

Bovine serum albumin (BSA).‡ BSA antigen for the groups injected at 4 or 16 weeks of age was prepared by suspending crystalline BSA in complete Freund's adjuvant (CFA)§ at a concentration of 5 mg of BSA per ml of adjuvant. For the group injected at 10 weeks of age, BSA was dissolved in saline and then mixed with an equal volume of CFA, forming a water-in-oil emul-

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†Pregnant rats were obtained from Carworth Farms, Inc., New City, Rockland County, New York.

‡Armour Pharmaceutical Co., Kankakee, Illinois, Lot No. Y20707.

§Difco Laboratories.

sion with a final concentration of 5 mg BSA per ml emulsion. Animals were injected in a hind foot pad with 0.1 ml of BSA preparation. A second injection was given into the opposite hind foot pad.

Sheep erythrocytes (SRBC). Antigen dose was 0.25 ml of 20 per cent washed sheep erythrocytes suspended in 0.9 per cent saline. Injection was into the lateral tail vein. A second injection 4 weeks later was identical to the first.

Flagella. Flagella from Salmonella typhosa serotype T-29 was prepared according to the method of Kobayashi,[2] as modified by Winebright and Fitch.[8] A suspension containing 10 μg flagella protein per ml was prepared in distilled water. Animals were immunized with 1.0 ml of this preparation injected into the lateral tail vein, a second injection was given 33 days later.

Flagellin. Soluble flagellin was prepared from an aliquot of flagella by acidification.[9] Concentration of flagellin was adjusted to 10 μg protein per ml with distilled water. Injections were as described above for flagella.

Bleedings. Under ether anesthesia, blood from the lateral tail vein was collected in plastic tubes and centrifuged after clot retraction. The sera were stored at -20° C.

Antibody Determination

Bovine serum albumin. Antibodies to BSA were measured by the passive hemagglutination technique.[8] Sheep erythrocytes were treated with tannic acid and then incubated with BSA. Starting with a 1:10 dilution of serum, double dilution was performed. Antigen-coated sheep erythrocytes were added to each tube. Antibody titer is expressed as the reciprocal of the highest serum dilution producing visible alteration in the settling pattern of tanned erythrocytes. Each preparation was tested against a known positive serum and a known negative serum before being used for titrations. An aliquot of each serum also was titrated after treatment with 0.1 M 2-mercaptoethanol (2-ME) for 30 minutes at 37° C. This procedure inactivates 19S hemagglutinins to sheep erythrocytes and agglutinins to Salmonella typhosa without apparent destruction of 7S antibody activity. For the sake of convenience, 2-ME sensitive antibody will be referred to as "19S" and 2-ME insensitive antibody will be referred to as "7S."

Sheep erythrocytes. Titrations were performed to measure agglutinating antibody against sheep erythrocytes. Sera were inactivated for 30 minutes at 56° C. Starting with a 1:10 dilution of serum, double dilution was performed. A 0.25 per cent suspension of thrice-washed sheep erythrocytes in saline was added to an equal volume of diluted serum in each tube. Antibody titer is expressed as the reciprocal of the highest serum dilution which produced an alteration in the settling pattern of erythrocytes. Aliquots of each serum also were titrated after treatment with 2-ME.

Flagella and flagellin. Antibody response to these flagellar antigens was measured by determining the agglutinin titer with the whole S. typhosa organism. Double dilution of serum was performed and a suspension of formalin-killed S. typhosa organisms added. Antibody titer is expressed as the reciprocal of the highest serum dilution producing grossly visible aggregation when the centrifuged bacteria were gently resuspended. Aliquots of each serum also were titrated after treatment with 2-ME.

Plaque-forming cells. The method of Jerne and Nordin[10] for demonstrating release of sheep cell hemolysins by single cells in agar plates was used with minor modifications.[11] Suspensions of spleen and lymph node cells were prepared from 5-week-old rats 4 days after intravenous injection with 0.25 ml of 20 per cent suspension of sheep erythrocytes.

Immunodiffusion. Precipitin antibody to BSA was demonstrated using the Ouchterlony technique.

Autopsies

At the completion of the experiments, animals were weighed and then killed under ether anesthesia. Spleen, liver, and thymus were weighed; samples of spleen, liver, thymus, and lymph node were fixed for histological preparations. In thymectomized animals, the anterior mediastinum was inspected and all tissue overlying the trachea, heart, and great vessels was carefully removed and weighed: Multiple tissue sections were prepared to permit evaluation of thymectomy. Most of the thymectomized animals used in this experiment had no residual thymic tissue; none appeared to have more than 25 mg.

RESULTS

Response to Bovine Serum Albumin

The response of rats to bovine serum albumin in complete Freund's adjuvant was impaired after neonatal thymectomy. Compared to non-thymectomized rats, fewer thymectomized rats responded with hemagglutinin or precipitin antibodies to BSA. None of the thymectomized rats responded with hemagglutinins or precipitins after receiving BSA in complete Freund's adjuvant at 4 and 8 weeks of age (Figure 1, Table 1). No thymic remnants were found in these animals. Their spleens had morphologic evidence of thymectomy as manifested by a reduction of lymphocytes in the white pulp, especially in the periarteriolar sheath. Of the non-thymectomized animals, 43 per cent produced hemagglutinins after a BSA injection at 4 weeks of age, and 86 per

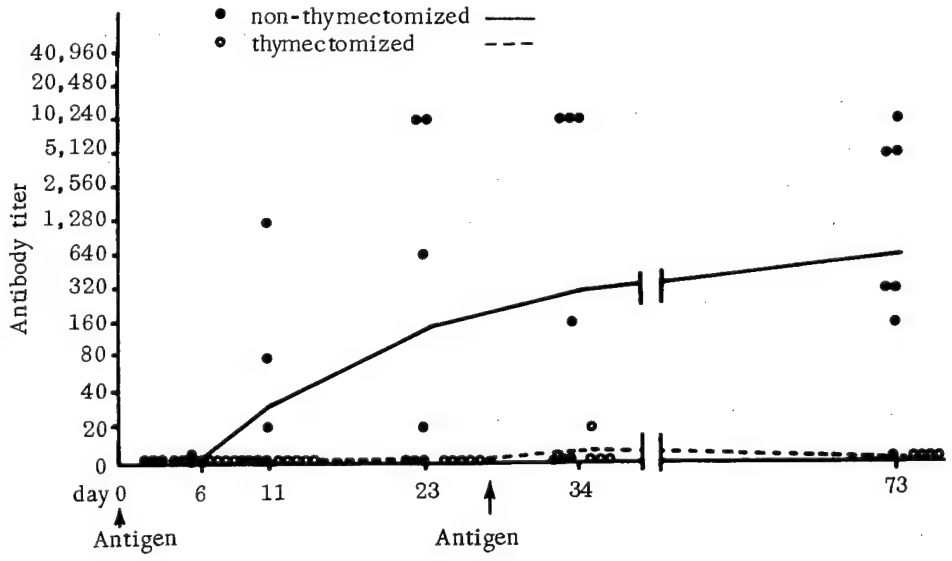

Figure 1. Passive hemagglutinin response of 4-week-old thymectomized and non-thymectomized rats after primary and secondary foot pad injection with bovine serum albumin suspended in complete Freund's adjuvant. Each point is the titer for a single animal. The lines represent the geometric mean titer for the group. A titer of 0 was arbitrarily assigned to those rats whose sera did not agglutinate coated red blood cells at the lowest dilution (1:20) tested.

Table 1

PERCENTAGE OF ANIMALS RESPONDING TO BOVINE SERUM ALBUMIN AND SHEEP ERYTHROCYTES

Procedure	Bovine serum albumin		Sheep erythrocytes	
	Primary	Secondary	Primary	Secondary
First exposure at 4 weeks of age				
Thymectomy	0	0	100	100
Non-thymectomy	43	86	100	100
First exposure at 10 weeks of age				
Thymectomy	80	80		
Non-thymectomy	100	100		
First exposure at 15 to 16 weeks of age				
Thymectomy	63	89	100	100
Non-thymectomy	83	100	100	100

cent responded after a second injection 4 weeks later. However, only 28 per cent of these non-thymectomized animals produced detectable precipitins. The hemagglutinin antibody that was present at 11 days was mainly of the 19S type, but 7S hemagglutinins predominated at 23, 34 and 73 days. Three non-thymectomized animals had high hemagglutinin titers, but only 2 of these formed precipitin antibody as measured by the immunodiffusion method. All of these thymectomized animals and non-thymectomized animals given BSA at 4 weeks of age were given sheep erythrocytes at 16 weeks of age.

Neonatally thymectomized rats given BSA prepared as an emulsion in complete Freund's adjuvant at 10 weeks of age had a lower mean hemagglutinin titer than did normal rats of the same age. There was a greater range of titer in the thymectomized group; these animals developed either no measurable titer, a persistently lower titer, or titers in the range of non-thymectomized rats (Figure 2). Hemagglutinins produced 7 days after BSA injection were mainly of the 19S type, whereas at 19 days and later, the hemagglutinin antibody was predominantly of the 7S type. Although 4 out of 5 thymectomized rats produced hemagglutinins, only 3 developed precipitins on immunodiffusion. Histologic preparations showed that two of these three animals had small remnants of the thymus, although these were not evident grossly.

Rats that had received sheep erythrocytes at 4 weeks of age were given BSA in complete Freund's adjuvant at 16 weeks of age. Thymectomized rats given BSA at this age had a lower mean titer and fewer of the animals responded, as compared with non-thymectomized animals (1:120 compared with 1:740 at 6 days after first injection, and 1:4800 compared with 1:19,000 at 6 days after a second injection 4 weeks later). Both thymectomized and non-thymectomized rats showed predominantly 19S hemagglutinins in the early primary response, and predominantly 7S hemagglutinins in the late primary as well as the secondary response. Three of 8 thymectomized rats failed to respond after a single injection. A fourth thymectomized rat produced a titer of only 1:40 at 6 days, and this fell to 0 before a second injection. Of the 4 thymectomized animals that responded with normal titers, 3 were found to have residual thymus. Two of these 3 rats

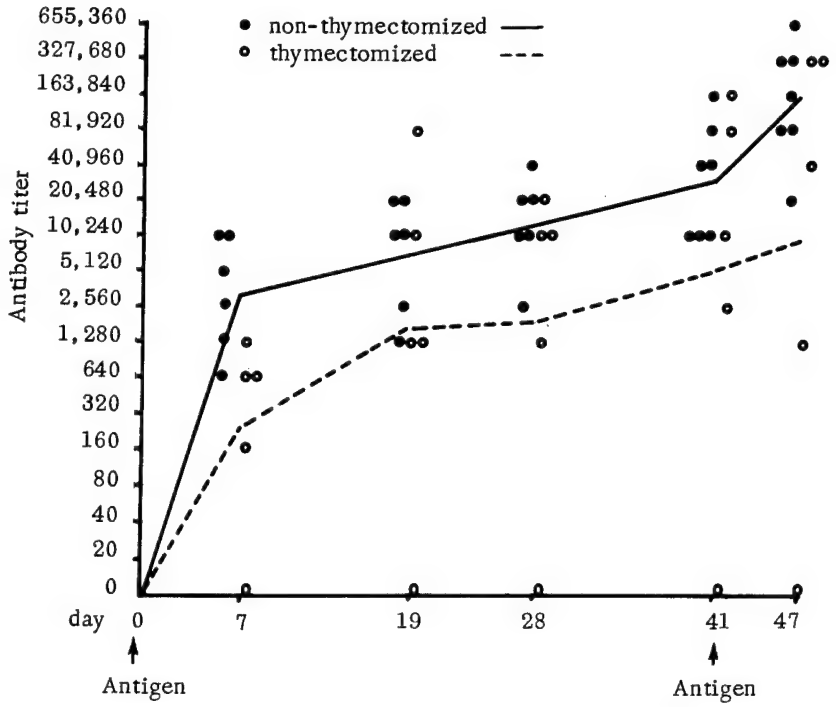

Figure 2. Passive hemagglutinin response of 16-week-old thymectomized and non-thymectomized rats after primary and secondary foot pad immunization with bovine serum albumin emulsified in complete Freund's adjuvant. Each point is the titer for a single animal. The lines represent the geometric mean titer for the group. A titer of 0 was arbitrarily assigned to those rats whose sera did not agglutinate coated red blood cells at the lowest dilution (1:20) tested.

also produced precipitating antibody to BSA. Eighty-three per cent of the non-thymectomized animals responded after a single injection with titers of 1:160 and above. After a second injection, all the non-thymectomized animals responded with titers of 1:10,000 or higher, but several thymectomized rats failed to respond or responded with a low titer (1:160).

All non-operated and sham-operated rats injected with BSA emulsion at 16 weeks of age produced precipitins as measured by double diffusion in agar gel. Serum from thymectomized animals either failed to develop precipitin lines or developed precipitin lines in the same manner as non-thymectomized animals. Precipitating antibody to BSA formed sharp lines in gel against BSA, 100 μg per ml, and was not inactivated by 2-ME treatment of the sera. It is, presumably a 7S immunoglobulin.[5] Some sera with high anti-BSA hemagglutinin titers had anti-BSA precipitating antibody while other sera with identical anti-BSA hemagglutinin titers did not. However, sera containing precipitins consistently had high hemagglutinin titers. Apparently at least 3 types of anti-BSA antibody molecules may be produced by the rat.

Response to Sheep Erythrocytes

Animals that received sheep erythrocytes as their first antigenic stimulation at 4 weeks of age had an identical response whether thymectomized or not. At 6 days after injection the antibody was mainly of the 19S type. A second injection of sheep erythrocytes given 28 days later resulted in the production of mainly 7S type of antibody, there being no appreciable difference

between thymectomized and non-thymectomized groups. By 80 days, the titers had fallen to less than 1:20 (Figure 3). Two of the 7 thymectomized rats were found to have small remnants of thymus.

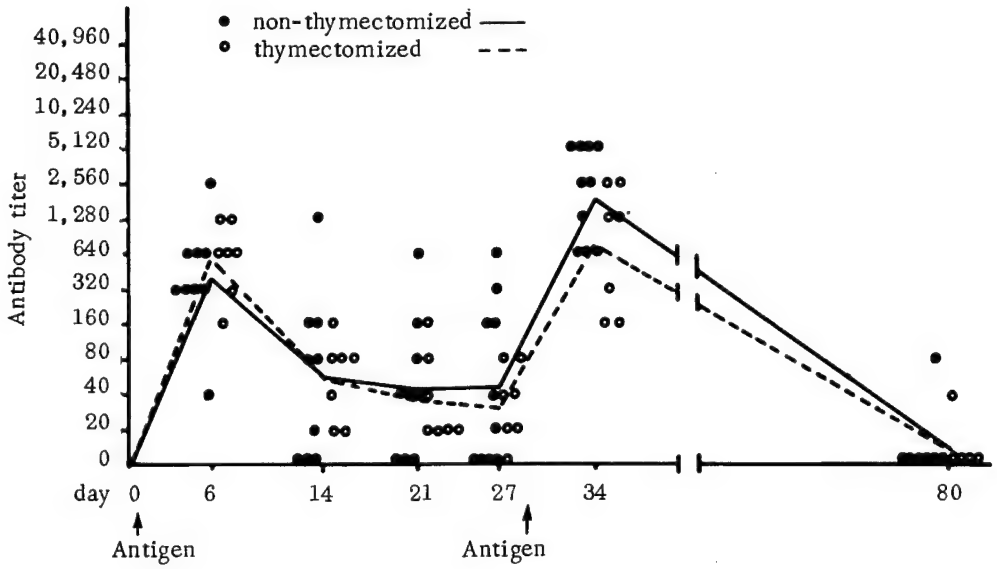

Figure 3. Hemagglutinin response of 4-week-old thymectomized and non-thymectomized rats after primary and secondary intravenous immunization with sheep erythrocytes. Each point is the titer for a single animal. The lines represent the geometric mean titer for the group. A titer of 0 was arbitrarily assigned to those rats whose sera did not agglutinate sheep erythrocytes at the lowest dilution (1:20) tested.

There was no difference between the thymectomized and non-thymectomized animals immunized at age 15 weeks with sheep erythrocytes. These animals had been immunized at age 4 weeks with BSA. The pattern of the response was the same as that observed after immunization at 4 weeks of age (Table 1). There was no essential difference in primary or secondary response whether the rats were 4 or 16 weeks of age when receiving the initial injection of sheep erythrocytes.

Compared with sham-operated rats, neonatally thymectomized rats had no suppression of plaque-forming cells in the spleen after immunization with sheep erythrocytes. Thymectomized rats had an average of 164 plaques per 10^6 spleen cells, compared with 181 plaques per 10^6 spleen cells in the sham-operated rats. Grossly, there was no evidence of thymus tissue at autopsy in these thymectomized rats, but sections of the spleens showed evidence of thymectomy. In one animal, several sections of mediastinum revealed tiny thymic remnants estimated to weigh less than 25 mg.

Response to Flagellar Antigens

The primary and secondary antibody response of thymectomized animals receiving S. typhosa flagella or flagellin at 11 weeks of age were not different from those of non-thymectomized rats (Figures 4 and 5). At 6 days after a primary injection, animals receiving flagella or flagellin had

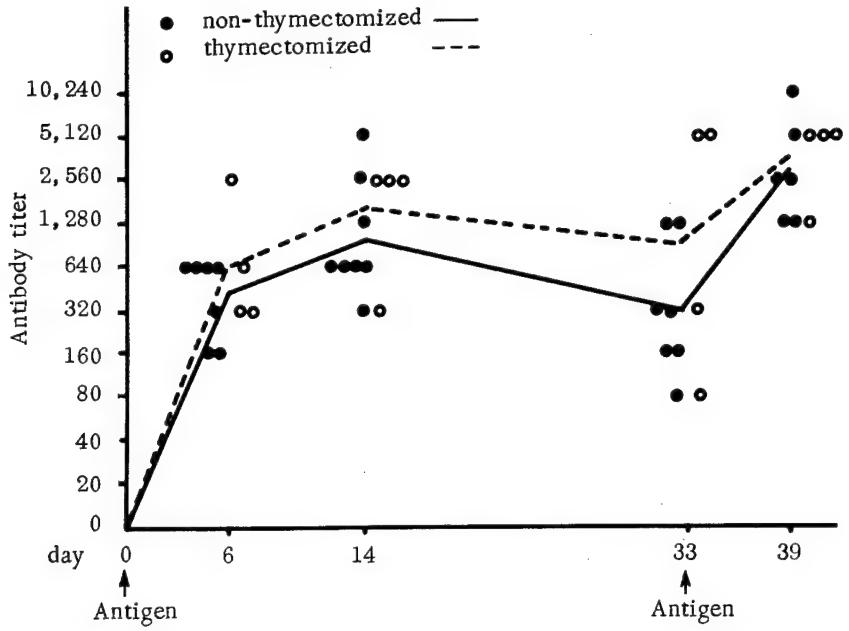

Figure 4. H-agglutinin response of 11-week-old thymectomized and non-thymectomized rats after primary and secondary intravenous immunization with flagella from Salmonella typhosa. Each point is the titer for a single animal. The lines represent the geometric mean titer for the group. A titer of 0 was arbitrarily assigned to those rats whose sera did not agglutinate bacteria at the lowest dilution (1:20) tested.

a high 19S agglutinin titer. At 14 days and 33 days after the primary injection, as well as 6 days after the secondary injection at day 33, most of the antibody activity was of the 2-ME insensitive type in animals that had received either flagella or flagellin. At autopsy, thymic tissue was found in one of 4 thymectomized rats immunized with flagella and in 2 of 5 immunized with flagellin. There was no difference in titer between these 3 animals and the other thymectomized rats.

DISCUSSION

In this study thymectomized rats receiving BSA had lower antibody titers than non-thymectomized animals when first immunized at 4, 10, or 16 weeks of age. The most marked depression of the response to BSA was found in the thymectomized rats that were 4 weeks old when first injected. These animals failed to respond with passive hemagglutinating or precipitating antibody either to the first injection or to a second injection at 8 weeks of age. Compared with non-thymectomized rats, there was a lower mean titer and a greater variation in titer of anti-BSA hemagglutinins in thymectomized rats first exposed to BSA at 10 or 16 weeks of age. This difference is the result of two factors; fewer thymectomized animals responded with measurable hemagglutinin titers, and several of those that did respond formed lower levels of antibody than non-thymectomized animals. However, some thymectomized animals formed hemagglutinin titers in the range of non-thymectomized animals, and several of these formed precipitins as well. At autopsy, remnants of thymus were found in about half of the animals that produced precipitins, and all of these also had high levels of hemagglutinin antibodies. Thymic remnants were

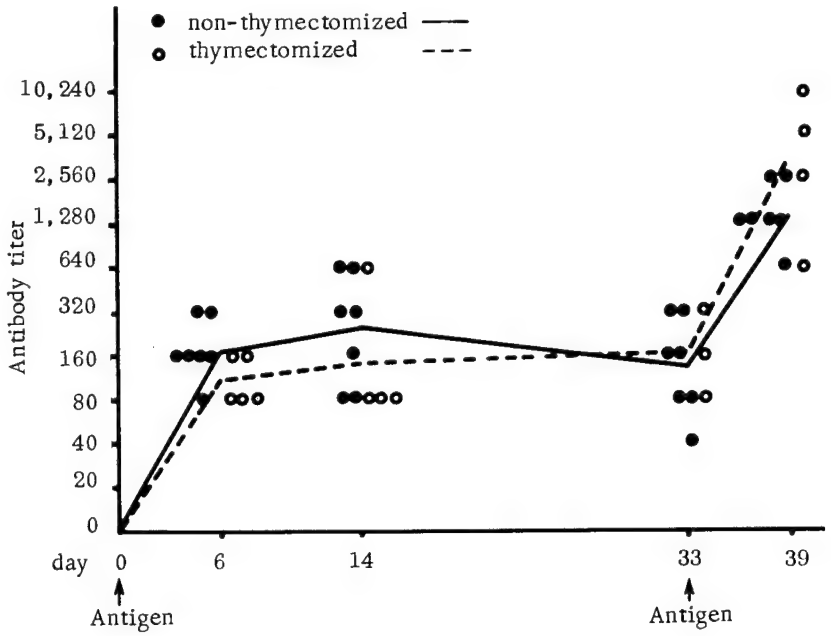

Figure 5. H-agglutinin response of 11-week-old thymectomized and non-thymectomized rats after primary and secondary intravenous immunization with flagellin from Salmonella typhosa. Each point is the geometric mean titer for the group. A titer of 0 was arbitrarily assigned to those rats whose sera did not agglutinate bacteria at the lowest dilution (1:20) tested.

not found in any animal which failed to produce precipitins or had a depressed or absent passive hemagglutinin titer. It is possible that all thymectomized animals which had measurable precipitin response to BSA had ectopic thymus or incomplete thymectomy.

Although antibody response to BSA was depressed regardless of the age at which antigen injection was begun, thymectomized rats had a normal response to sheep erythrocytes as measured by hemagglutinin titers and number of plaque-forming cells in the spleen. Even animals that failed to produce hemagglutinins and precipitins against BSA developed a normal primary and secondary response after immunization with sheep erythrocytes. Since BSA is a purified protein antigen, and sheep erythrocytes constitute a complex particulate antigen, the difference in responsiveness of thymectomized rats to these antigens could be related to the physical state of the antigen. However, 11-week-old thymectomized and non-thymectomized rats showed no difference in the primary or secondary response to the soluble or particulate form of the flagellar antigen of S. typhosa. The variables of age, dose, and route of injection merit further study, but on the basis of the present work, it would appear that the physical nature of the antigen is not the decisive factor in determining whether antibody production is depressed or normal in neonatally thymectomized rats.

It appears that the antibody response of the rat to BSA does not mature as rapidly as that to sheep erythrocytes. Rats given sheep erythrocytes at 4 weeks of age had almost the same antibody titer as rats first immunized at the age of 15 weeks. Rowley and Fitch[12] also found this to be true for plaque-forming cell and antibody responses of rats given sheep erythrocytes. However, the response to BSA was less in 4-week-old control animals than in 16-week-old rats.

Thymectomy appears to have a greater effect on the less mature responses to BSA than on the more mature response to sheep erythrocytes.

In other species of animals the immunological response does not develop simultaneously for all antigens. The ontogeny of the immune response has been studied most thoroughly in the sheep. The lamb, which has a gestational period of 150 days, is a relatively mature animal at birth. The fetal lamb can respond to some antigens, including bacteriophage ØX174 and ferritin, before the sixty-seventh day of gestation. It cannot respond to ovalbumin until the 125th day of gestation. Even at 42 days after birth, the lamb still does not respond to S. typhosa, diphtheria toxoid, or BCG, although adult sheep respond well to these antigens.[13] It appears that the state of immunologic reactivity is a stepwise phenomenon which develops at different times for different antigens.

In normal mice, the response to hemocyanin apparently matures early, since circulating antibody appears as early as 1 week after birth after injection with this antigen.[14] Neonatally thymectomized mice are capable of responding in nearly normal fashion to this antigen.[7] Thus in the mouse, thymectomy does not impair antibody production in a system that matures early.

Thymectomy does not affect antibody response in all species of animals in the same way. Although the response of the rat to sheep erythrocytes and typhoid "H" antigen is not impaired by thymectomy, the antibody response to both antigens is depressed in thymectomized mice.[7,4] Histologic studies by Waksman et al.[15] demonstrate that neonatal thymectomy of the rat results in a disappearance of small lymphocytes from the splenic white pulp and periarteriolar reticular structures, while the germinal centers and perifollicular zones remain essentially normal. In thymectomized mice, however, Miller[16] observed a conspicuous deficiency of germinal centers and only a few plasma cells in the lymph nodes and spleens. These data suggest that the histologic consequences of neonatal thymectomy are greater in the mouse than in the rat.

Immunologic maturity varies according to species, antigen and dose of antigen.[14] Removal of the thymus appears to arrest the development of the lymphoid tissue at the stage prevailing at the time of thymectomy.[17] Neonatal thymectomy seems to impair antibody production to a greater extent in systems that reach immunologic maturity at a later time.

The degree of suppression of the immune response of neonatally thymectomized rats to BSA was not as marked at 16 weeks of age as at 4 weeks of age. This indicates that continued development of immunological capabilities was possible even in thymectomized rats although the rate of development was not as great as in intact rats. Similar findings have been reported for the mouse. Brooks[18] was unable to demonstrate inhibition of antibody response to sheep erythrocytes or flagellar antigen of Salmonella in neonatally thymectomized mice immunized at 9 weeks of age although the response to these antigens was impaired in neonatally thymectomized mice immunized at a younger age.[19,16] Moreover, neonatally thymectomized mice recovered less rapidly than intact mice from the physiological hypogammaglobulinemia present in the first few weeks after birth. However, most thymectomized mice that survived past 6 weeks of age had normal globulin levels.[19] These observations suggest that the thymus exerts a quantitative influence on the immunoglobulin forming system; immunological responsiveness can develop after thymectomy, although at a slower rate than normal.

The authors wish to acknowledge the technical assistance of J. Robinson, Y Hamada, Karen Craft, and T. Guinsaotao.

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THREE-DIMENSIONAL IMAGE RECONSTRUCTION IN SCANNING AND RADIOGRAPHY*

By

P. V. Harper

The past few years have seen a few highly significant steps forward in the scanning field in the realm of cameras and isotope generators. The most highly developed examples of these are, respectively, the Anger Scintillation Camera and the molybdenum-99–technetium-99m generator, both of which are now in use in a number of institutions. Together, these developments have increased the information available in scintillation scans by a very large factor, so that processing techniques that were unthinkable five years ago now become feasible.

The Anger Camera,[1] a relatively old invention as scanning goes, was described approximately ten years ago and was exhibited at the 1958 Geneva Conference on Peaceful Uses of Atomic Energy. The justification for discussing its capabilities at the present time is that only in the past year or so has it become commercially available in usable form, due to the efforts and foresight of the Nuclear Chicago Company. Briefly, this camera is a device able to detect by means of an array of photomultiplier tubes and associated circuitry the position of a scintillation event in a large diameter (11 inch) thin (1/2 inch) NaI crystal to within considerably less than a centimeter, speaking statistically, so that the principal resolution loss lies in the collimation. The advantages of this device lie in simultaneous viewing of the entire area of interest and an immensely increased sensitivity, while retaining reasonable resolution. Although the Anger Camera will probably never completely displace the moving detector system, especially where fine resolution is critical, it clearly opens new avenues, especially where sensitivity is important, as in dynamic studies.

Technetium-99m first came to the attention of clinically-oriented people at about the same time as the Anger Camera was invented,[2] as a contaminant of the ^{132}Tc-^{132}I generator manufactured by Brookhaven National Laboratory. Mr. Powell Richards of the Nuclear Engineering Division at Brookhaven was stimulated to investigate this material, with the resulting development of the familiar ^{99}Mo-^{99m}Tc generator in which the 6-hour pertechnetate-99m is eluted virtually pure with dilute acid or normal saline from the 67-hour molybdate-99 parent adsorbed on a small aluminum oxide column. The short half life, absence of primary particle radiation, and clean 140 keV γ, make this material ideal from a physical point of view, permitting multi-millicurie isotope administration with trivial radiation dosage to the patient. Its biological properties as pertechnetate make it very useful as an extracellular fluid tag in brain tumor scanning; its ionic properties make it a good thyroid scanning agent; and its chemical flexibility makes it usable as a liver, spleen, bone marrow, placenta, and kidney scanning agent. Its 140 keV gamma is almost as easily collimated as ^{125}I radiation, and, in the present context, almost all of the 140 keV γ energy is photoelectrically absorbed in the thin (1/2 inch) crystal of the camera, giv-

*This paper was presented before the XIth International Congress of Radiology, Rome, Italy, September 1965 and appears in the Proceedings of this Congress.

ing imaging properties not appreciably degraded from those, for instance, of ^{203}Hg, which emits a 279 keV gamma. These advantages have become obvious during the past few years and its clinical use is rapidly increasing.

Although use of the Anger Camera and ^{99m}Tc has increased the count rate available in scanning procedures by approximately two orders of magnitude, it was the proposal of tomographic or section scanning by Kuhl[3] which focused attention on the possibilities of exploiting these gains more fully. In his procedure of transverse section scanning, Kuhl uses linear picture elements derived from scanning information to build up an image of remarkable detail and resolution. In this technique, the desired slice, or section, is scanned linearly along the edge in the plane of the section with registration of the observed counts as linear elements parallel to the detector axis. Repetition of this process from many angles builds up a picture where the linear elements cross and reinforce each other, producing pictures of remarkable detail and clarity. Regions where the linear elements do not cross and reinforce are eliminated by background erase.

It is obvious that full three-dimensional mapping of an isotope distribution in the head, for instance, might be accomplished by repetition of this process, performing section scans at successive levels. Although this could be done without undue increase in scanning time by multiplying the scanning heads and read-out units, it occurred to us that the available commercial Anger Camera could be used to accomplish exactly this result, by recording a substantial number of pictures (say 50) from as many different angles in a few seconds each.[4] In reconstructing the original isotope distribution in the object from this information, recorded most economically on a series of photographs, a great number of possible options exist. The films may be scanned in strips, recording individual cuts using Kuhl's two-dimensional read-out, or this procedure may be carried out on the original information from the camera, properly channeled and recorded on tape. A more valuable presentation, however, might be obtained by carrying out the procedure in all planes simultaneously, thus creating a full three-dimensional display of the isotope distribution, which might then be dissected optically with complete freedom and flexibility to display any plane in any orientation, or indeed the full three-dimensional image from any angle.

To accomplish this, the principle used by Kuhl may be extended. This is in fact a generalization of the principle used in conventional body section radiography. The recorded plane images are projected into a space (image space) from many directions and reinforce each other where they are in register, producing a display that may be visualized by insertion of a translucent screen into the image space. To show the full three-dimensional image, the screen may be rotated rapidly, allowing the eye to synthesize the full display. As other options using eye integration, the image may be created with a single rotating optical system by projecting rapidly a series of pictures into the image space, or a single stationary optical system may be used to produce a rotating image that can be viewed through a rotoscope.

In order that the pictures may be in register in the image space, it appears that they should be projected from the same angle with the same degree of convergence or divergence as the gamma rays originating from the original object. Figure 1 shows one possible approach. Using a parallel hole collimator, the pictures are recorded on successive frames of 35 mm film as the subject is rotated about a vertical axis for a small fixed angle between frames. These are then projected back into the image space using a paraboloid zone mirror to parallelize the diverging rays from the point-source, which functions optically in a manner similar to a pin hole,

but provides a much greater degree of illumination. Figure 2 shows a more detailed sketch of this apparatus. This approach is equivalent to reduplicating Kuhl's method.

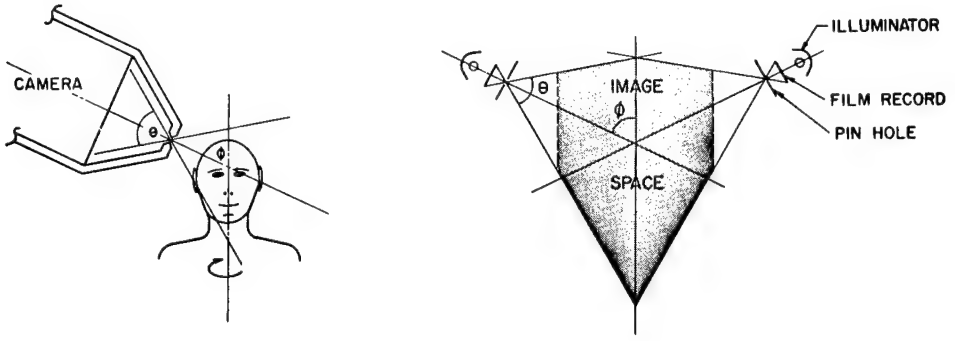

Figure 1.

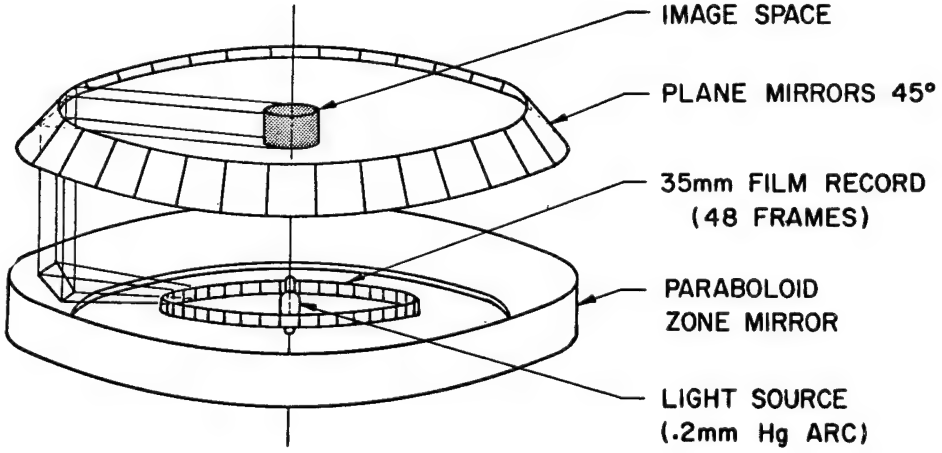

Figure 2.

Figure 3 demonstrates the possibilities of using a pin-hole collimator for recording the information. Here the display mechanics become ridiculously simple, the only serious problem being the amount of light available through the pin holes.

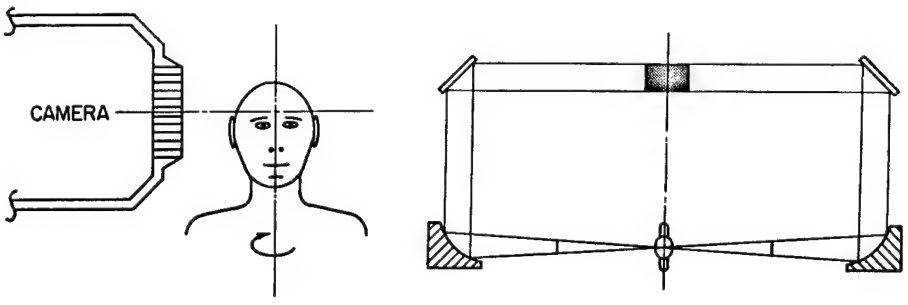

Figure 3.

The possibilities of using such a system in connection with individual cineradiographic frames occurred to us. It became evident upon close examination that it was immaterial whether the light rays carrying the images converged or diverged, and that images remained in register (or rather reverse register) without distortion depending on whether the rays converged or diverged, as long as the angle was the same (Figure 4).

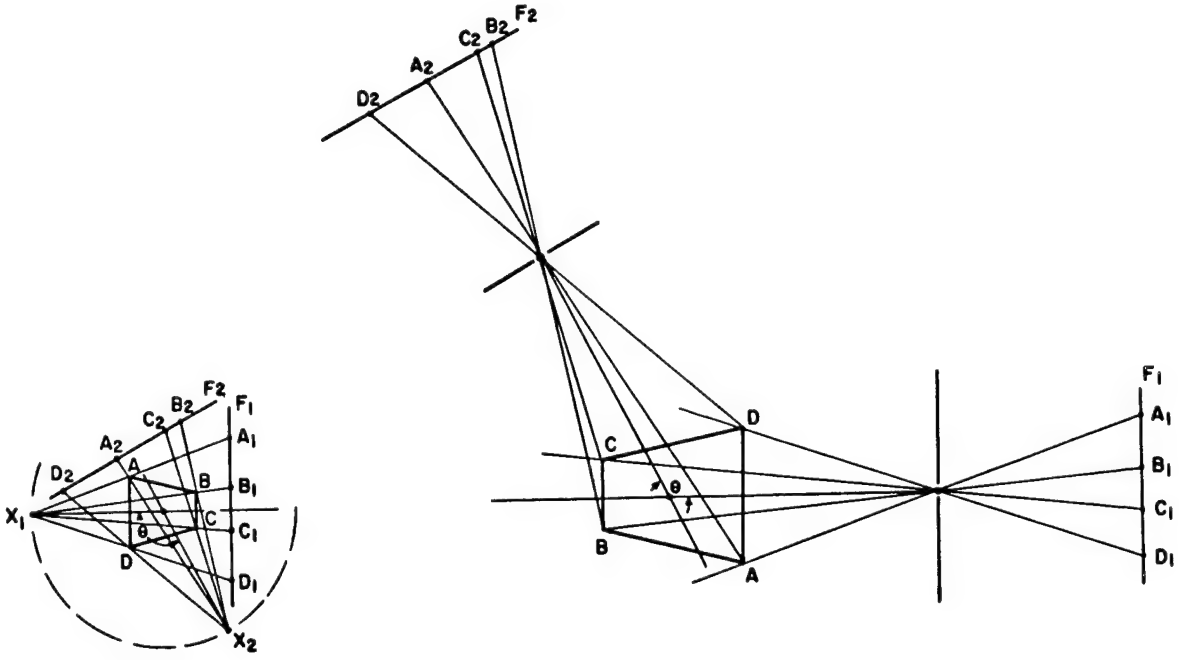

Figure 4. Left: Object ABCD is shown with projections on films F_1 and F_2 from X-ray source at positions X_1 and X_2 respectively. Right: Images of films F_1 and F_2 are projected through pin holes located at same positions relative to films as X_1 and X_2. The projections come into register forming a real three-dimensional reversed image. The number of projections which can thus be superimposed is limited only by practical considerations.

As far as concrete efforts are concerned, we have been vigorously pursuing the parallel ray options shown above. We have shown that images can indeed be projected from a point source mercury arc without too serious degradation of the image quality, and we are in the process of constructing the paraboloid zone mirror to test the practicality of these speculations. The utility of three-dimensional images has been strikingly demonstrated by R. L. deMontebello,* using somewhat different techniques, and we are confident that such information processing will prove of great clinical usefulness in selected cases.

In summary, the art of scanning during the past few years has entered a new phase, where in terms of image quality (quantum statistical fluctuation and modulation transfer function), it begins to be possible to think of scanning qualitatively in the same terms as conventional radiography, since ideas and techniques developed in one field frequently appear to have applications in the other. One outgrowth of this is the possibility of a three-dimensional display produced by a generalization of the principles of body section in radiography.

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IMAGING TECHNETIUM-99m COMPOUNDS WITH THE GAMMA SCINTILLATION CAMERA, AND THE USE OF THIS SYSTEM TO AVOID THE DISTORTION OF RESPIRATORY MOTION IN RADIOISOTOPE SCINTIPHOTOGRAPHY*

By

A. Gottschalk, F. F. Jiminez, C-t. Lu, R. N. Beck, and P. V. Harper

When the high count rates that result from the use of technetium-99m are combined with the sensitivity of the gamma scintillation camera,[1,2] studies of excellent quality (scintiphotographs) are possible with exposure times of less than one minute per view. This system can thus be used to extend the clinical scope of radioisotope scanning. In this communication, particular emphasis will be placed on the loss of resolution caused by respiratory motion, and the efforts made to correct this problem.

The Nuclear Chicago Pho/Gamma scintillation camera, which uses ratio correction circuits to increase the acceptable window widths and thereby improve the count rate, has been employed in Argonne Cancer Research Hospital for the past twelve months. The readout has been modified by placing a piece of diffusing glass 1-1/4 inches from the oscilloscope face to smooth the image. The camera is well suited for the clean 140 keV gamma ray of technetium-99m, since the 1/2 inch thick sodium iodide crystal is 100 per cent efficient for this gamma energy. Because of its short physical half life of six hours, and the absence of primary particle radiation, millicurie amounts of this radionuclide can be administered to the patient, and, although the 140 keV gamma ray of technetium-99m is penetrating enough to be useful with deep-seated organs (half value layer equals 4.5 cm of tissue), it can still be readily collimated. A suitable low energy high resolution multiaperture collimator is available for use with the camera.

The expanding scope of technetium scintiphotography is illustrated in Figure 1, which demonstrates the wide variety of examinations that can be made with the numerous forms of technetium-99m.[3,4] Although comparable studies are possible with conventional scanning equipment, they take more time. To emphasize the value of decreasing the imaging time, the remainder of this communication will be devoted to a consideration of the vexing problem of respiratory motion in scanning organs such as liver and spleen, and the solution of this problem by the use of technetium-99m sulphur colloid with the scintillation camera.

The magnitude of the resolution loss caused by respiration has been studied previously in this institution by scanning a moving Siemens star phantom with a variable speed research scanner,[5] a conventional 3-inch rectilinear scanner (Magnascanner), and the scintillation camera.[6] The phantom was mounted on a platform with a back-and-forth motion simulating respiration. In addition, a 2-inch layer of unit density scattering material was placed on top of the phantom to approximate radioactivity in a large organ such as the liver. The effect of motion on the Siemens star pattern is seen in Figure 2. Quantitation of the loss in resolution caused by motion

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*This report is taken from a paper that was presented before the 7th International Symposium on Radioactive Isotopes in Clinical Medicine and Research, Bad-Gastein, Austria, January 1966, and appears in Strahlentherapie. The work was supported in part by U. S. Public Health Service General Research Support Grant 1-SO1-FR-05367-01.

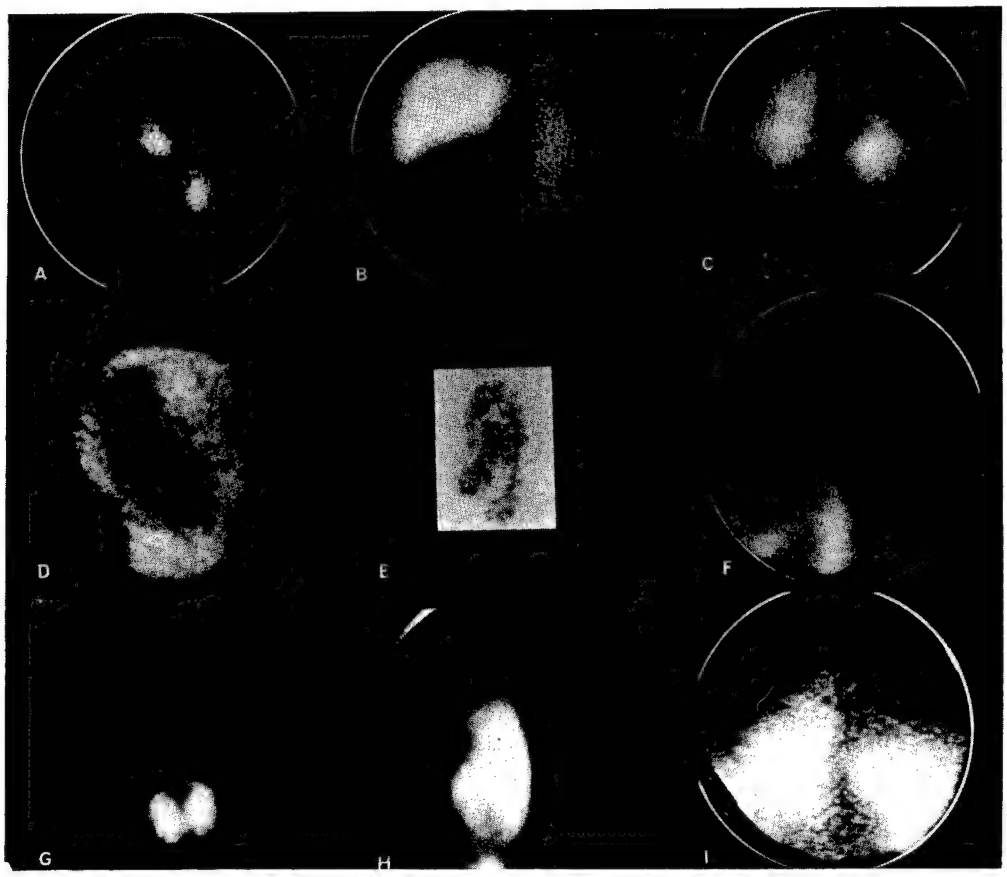

Figure 1. Clinical scope of ^{99m}Tc scintiphotography. A—Lateral scintiphotograph of the knee of a 20-year-old male with hemolytic anemia. The increase in bone marrow is demonstrated with 3 millicuries of ^{99m}Tc sulfur colloid and a 10-minute exposure. B—Hepatosplenomegaly in a 6-year-old child with leukemia. The scintiphotograph was made in 1 to 2 minutes with 400,000 dots collected. An intravenous dose of 0.5 millicuries of ^{99m}Tc sulfur colloid was administered. C—Posterior view of the kidneys 1 hour after a 1 millicurie dose of ^{99m}Tc iron complex. The mass in the upper and lateral aspect of the right kidney was a renal cyst. The exposure took about 4 minutes with 300,000 dots collected. D—Anterior view of a gravid uterus with the placenta in the left upper quadrant. A dose of 1 millicurie of ^{99m}Tc albumin was used for this 4-minute exposure. E—Lateral view of the heart and great vessels. This frame is part of a time sequence strip of pictures. The left ventricle, left atrium, and aortic arch are seen. The study was made with 1-1/2 millicuries of ^{99m}Tc sulfur colloid (for subsequent liver scan) injected via the anticubital vein. The exposure took ONE SECOND. F—Lateral view of the head showing a parietal brain abscess. This 3-minute exposure was made 10 minutes after intravenous injection of 2 millicuries of ^{99m}Tc pertechnetate. 100,000 dots were collected. G—Increased thyroid trapping in Grave's disease is clearly demonstrated by this 1-minute scintiphotograph taken 3 to 4 minutes after injection of 1 millicurie of ^{99m}Tc pertechnetate. Note the salivary gland uptake as well. H—Posterior scintiphotograph of the lungs of a 40-lb dog taken with a single pinhole collimator moved away from the animal to expand the field of view of the camera. The area from head to bladder is visible. A 5-millicurie dose of ^{99m}Tc macroaggregated albumin was used. The exposure time was 2 minutes, 40,000 dots were collected. The decreased uptake on the left is due to beginning rejection of a lung homotransplant. Quantitation of blood flow is readily achieved by placing a thin lead strip over one lung, absorbing the 140 keV gamma rays from that side, and recording the counts from the other lung using the camera as a scaler. The process is repeated for the other side, and a ratio of right to left lung perfusion is obtained. I—^{99m}Tc inhalation lung scan. ^{99m}Tc pertechnetate is vaporized from a hot filament and inhaled. This anterior view was made in 1 minute with a 350 microcurie dose about 10 minutes after inhalation.

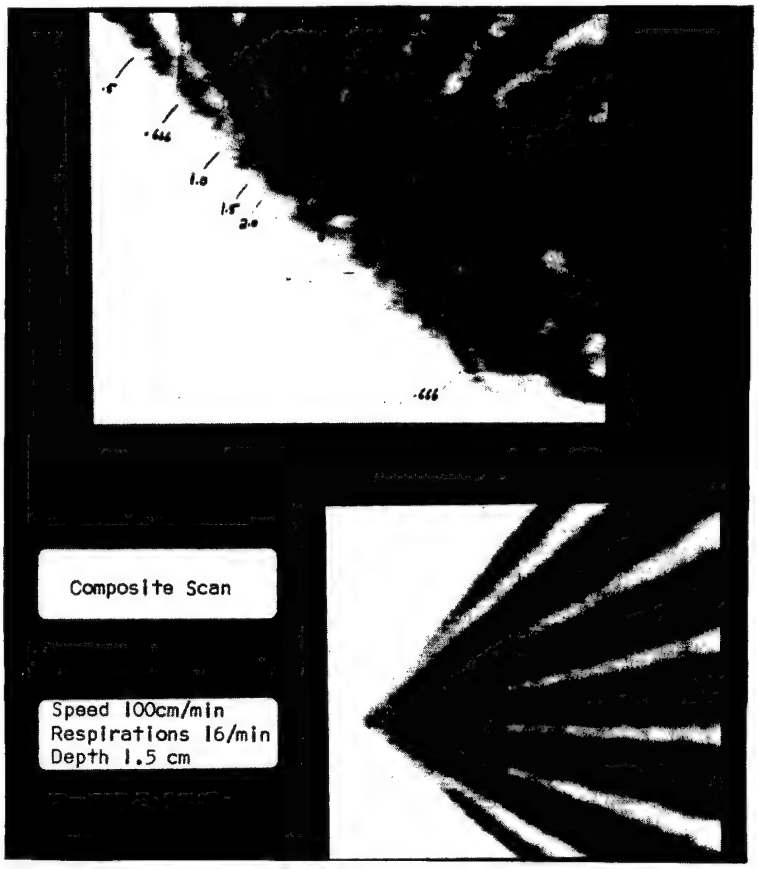

Figure 2. The effect of simulated respiration on the Siemens star phantom. TOP—a composite made by placing two scans of the moving phantom adjacent to each other. The direction of motion was vertical for this study. The phantom was oriented with the central ray in the direction of motion for the first scan, and perpendicular to this for the second scan. The numbers indicate the frequency (ν) of the rays in lines/cm. The extensive distortion caused by the motion of normal respiration is evident when the composite is compared with a scan of the stationary phantom seen at the BOTTOM to the RIGHT.

shows that the resolution distance was increased threefold with the research scanning system, and doubled for both the gamma scintillation camera and conventional rectilinear scanner. The final resolution with motion, however, was found to be best for the gamma camera, intermediate for the research scanner, and worst for the conventional rectilinear scanner.

Even though the scintillation camera—a motionless instrument—provided the best images of the moving phantom, the resolution loss was still significant. In an effort to eliminate completely distortion caused by respiration, the sensitivity of the scintillation camera was utilized with technetium sulphur colloid. Like other colloidal materials, this agent is taken up by the reticuloendothelial system, and therefore has been used for liver, spleen, and bone marrow scanning at the University of Chicago.

The method of preparation as well as the preparation of the remainder of the technetium-99m compounds used at this institution has been presented in detail elsewhere.[7,8]

A two- to three-millicurie dose of technetium sulfur colloid is routinely used for liver scan-

ning, the liver dose being less than 1 rad from this procedure. With a 30 per cent window (using ratio correction circuits) count rates of 500,000 to 700,000 per minute are achieved with the conventional scintillation camera (Nuclear-Chicago Pho/Gamma) and the 1-3/4 inch low energy multiaperture collimator. Since 400,000 dots are routinely collected per view, a single exposure takes less than 1 minute. A cooperative patient is able to hold his breath for this interval, thus obviating motion altogether. When the patient cannot cooperate for so long, an alternative method is used. The patient is asked to hold his breath in full expiration. [This position has been chosen, because it has been noted that varying degrees of inspiration produce distortion of the liver (see Figure 3).] The exposure is started when the breath is held. By a hand signal, the patient

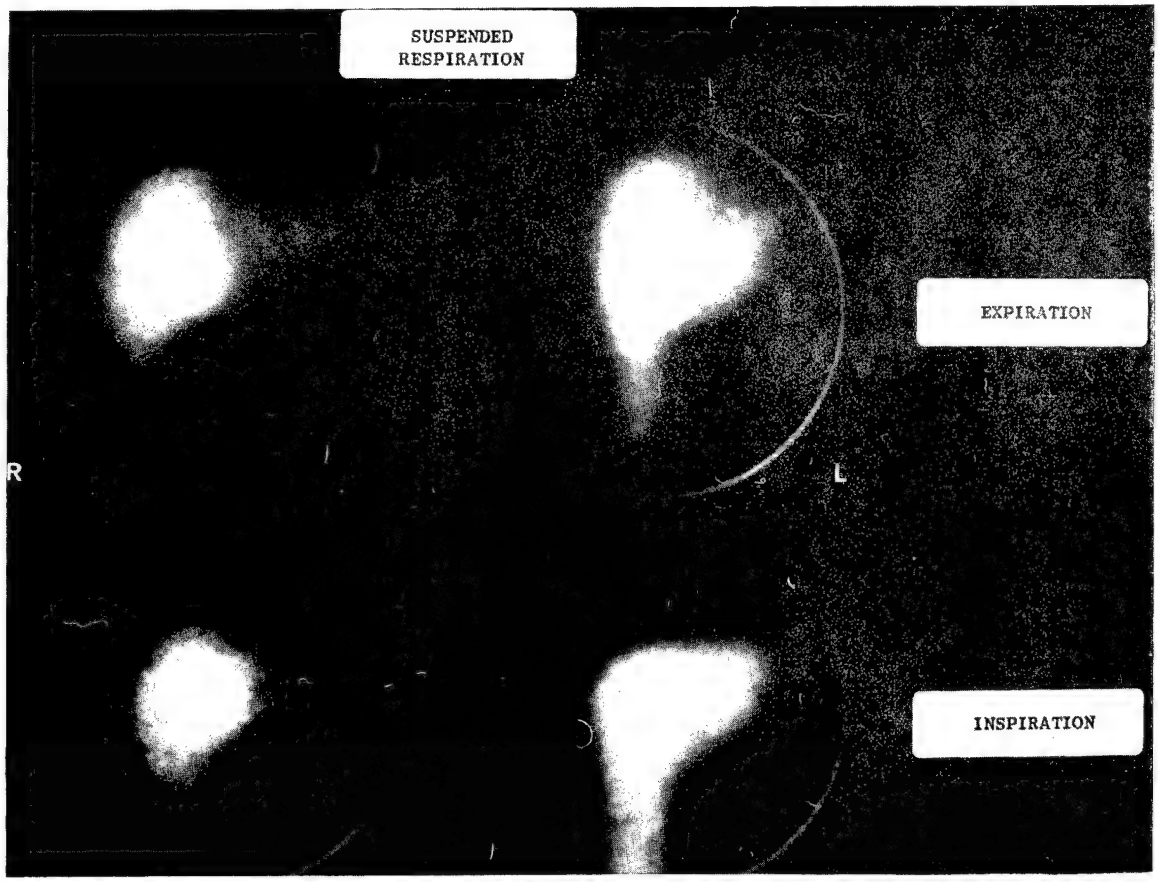

Figure 3. The effect of respiration on hepatic contour. TOP ROW—Two normal livers examined in full expiration with breath holding (see text). A dose of about 3 millicuries of ^{99m}Tc sulfur colloid was injected 15 minutes prior to the onset of the examination. It takes less than 1 minute of exposure time to obtain the 400,000 dots collected per view. BOTTOM ROW—The same patients examined in full inspiration. The marked change in shape that can result from respiration is obvious.

signifies his desire to breathe, and the exposure is stopped. When the patient is comfortable, he again holds his breath in complete expiration and the exposure is continued. The procedure is repeated until the desired number of dots has been accumulated on the scintiphotograph. This technique can be used with all but extremely ill subjects.

Because the camera images isotopes without mechanical motion, the respiratory motion artifact is not readily apparent on routine liver studies, being manifest only by an indistinctness of the edge of the liver. (See Figure 4.) The image is improved, however, when the technique of suspended respiration is used, for the edges of the liver are more sharply defined, and small lesions, previously distorted by respiratory motion, are more apparent (see Figures 5-7).

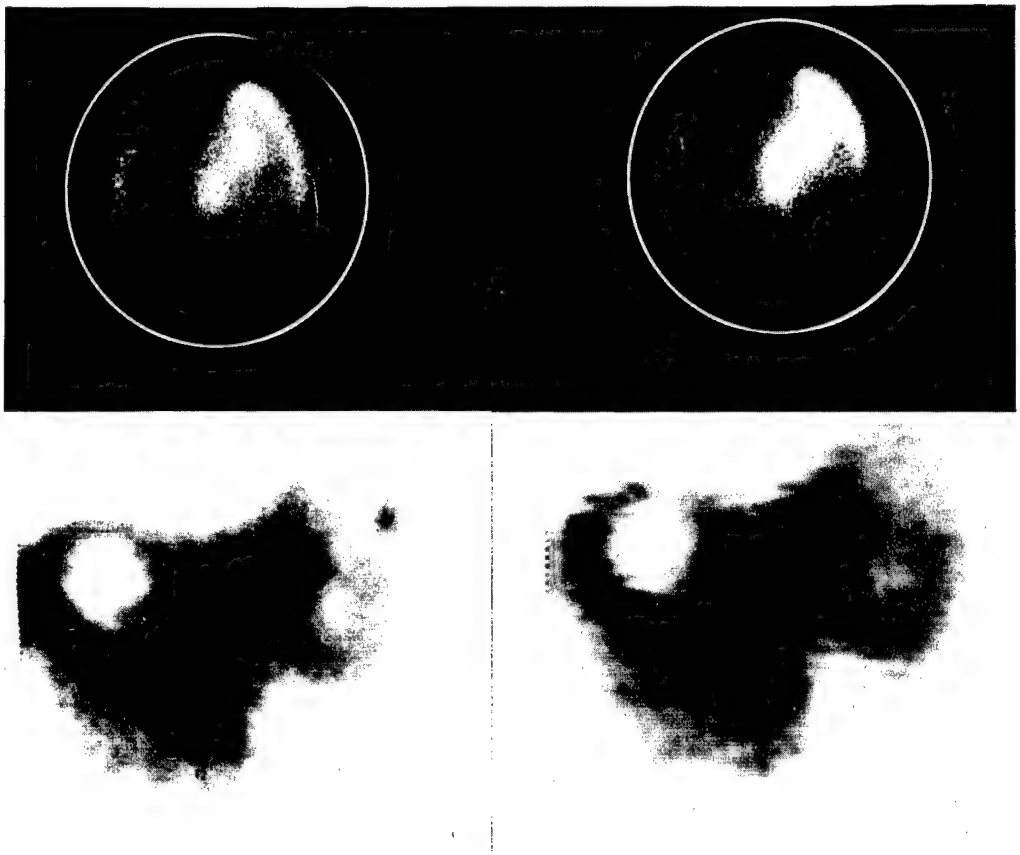

Figure 4. The qualitative appearance of the motion artifact on a liver phantom. The four studies were all done sequentially, so that the amount of ^{99m}Tc in the phantom was essentially constant. TOP LEFT—Scintiphotograph of the stationary phantom. TOP RIGHT—Scintiphotograph of the moving phantom. The direction of motion was vertical with a total distance of travel of 1.5 cm, and a rate of 12 per minute. This is comparable to a normal patient lying supine, and breathing quietly. Notice that the only change caused by motion is a loss of sharpness about the edges. BOTTOM LEFT—Scan of the stationary phantom made with a scan speed of 100 cm/min, using a 253-hole collimator 1.375 inch thick with 2.5 inch focal distance, and a 0.436 inch diameter of view at the focal depth. BOTTOM RIGHT—Scan of the moving phantom with the same rate and excursion of respiration as in the moving scintiphotograph. A severe edge distortion is clearly seen.

DISCUSSION

An alternative method of avoiding the respiration motion artifact has been used successfully by Harper et al.,[9] who temporarily paralyzed the right phrenic nerve by a cervical block with local anesthesia. The principal disadvantage of this method is that it causes a marked augmen-

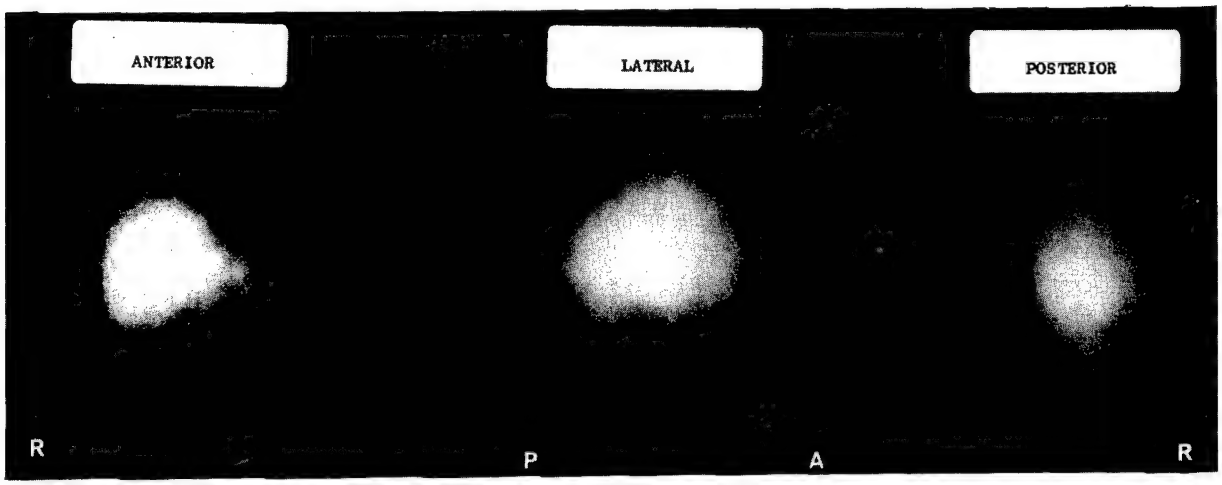

Figure 5. Hepatic scintiphotographs using the technique of suspended respiration on the anterior view. Note the sharp contours of the liver compared with the previous phantom study. Multiple metastases are shown. The medial aspect of the right lower lobe has several small lesions. These are the type that are usually obscured by motion. In addition, the value of multiple views of the liver is shown. Several lesions are demonstrated on both lateral and posterior projections that would be missed if only the frontal scintiphotograph was obtained. A 3 millicurie dose of ^{99m}Tc sulfur colloid was injected about 15 minutes prior to these pictures which took about 1 minute per exposure with 400,000 dots collected per view.

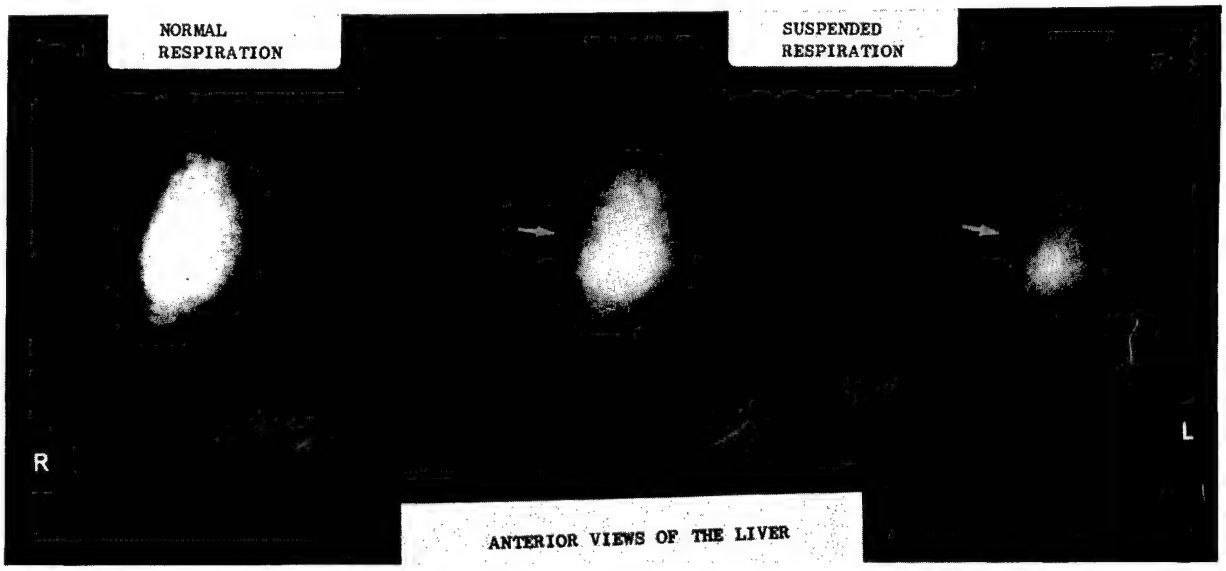

Figure 6. This patient had an unusual history. At a previous celiotomy, a diagnosis of gastric carcinoma with widespread metastases was made. She received combined therapy with colchicine and radiation, and showed a marked improvement. Prior to a second surgical exploration, these liver scintiphotographs were obtained. TOP LEFT—Anterior view with normal respiration after 2 to 3 millicuries of ^{99m}Tc sulfur colloid. 400,000 dots were collected in less than 1 minute. Note that the right lateral margin is essentially normal. No uptake is seen in the left lobe. TOP CENTER and TOP RIGHT—Anterior views with suspended respiration taken simultaneously, but with different lens apertures between the oscilloscope face and the film readout. A definite lesion is seen along the right lateral margin (arrows). At surgery the left lobe was minute, and believed to represent an instance of embryonic vascular compression by the hepatic ligaments with resultant fibrosis due to ischemia. A scar—about 2 cm in diameter in its frontal aspect—was present along the right lateral margin (previous tumor site?) corresponding to the lesion demonstrated on the scintiphotographs. No metastatic carcinoma could be identified in the abdomen.

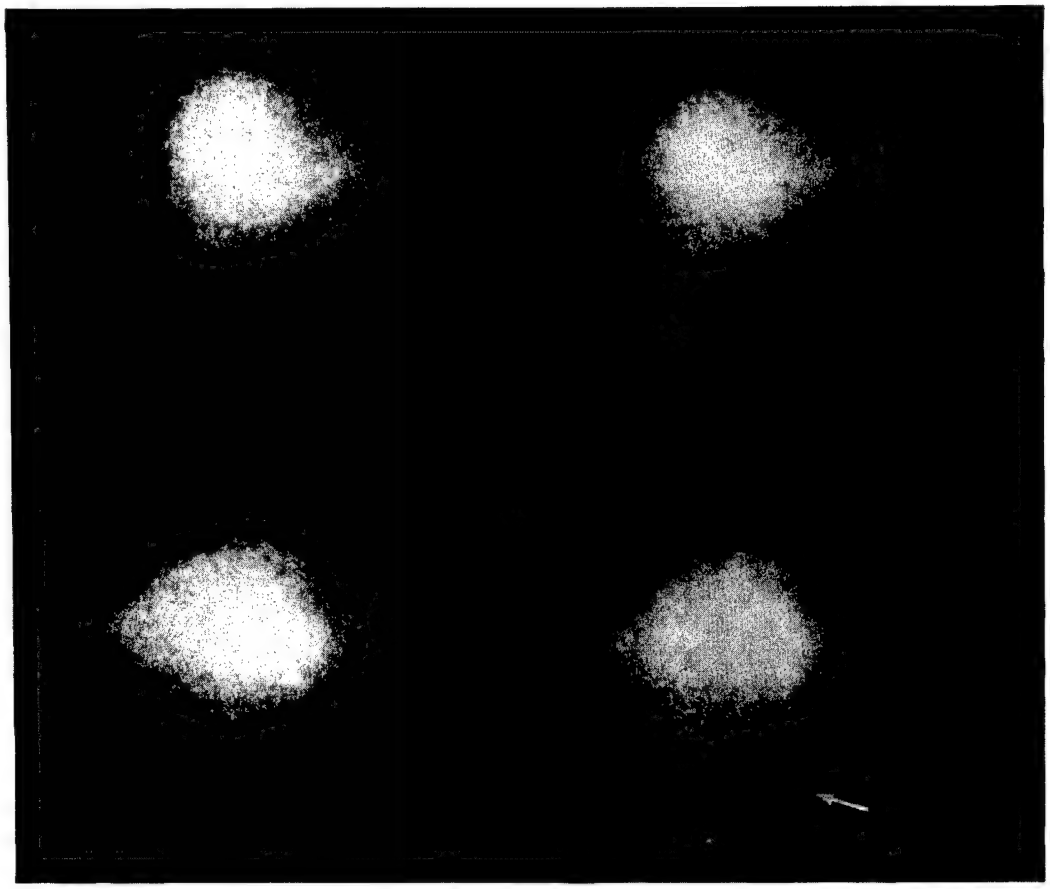

Figure 7. These liver studies were made with 3 millicuries of ^{99m}Tc sulfur colloid and exposure times of about 1 minute. 400,000 dots were collected per view. TOP LEFT—Anterior view with respiration, considered normal. TOP RIGHT—Anterior view with respiration suspended, also believed normal. BOTTOM LEFT—Right lateral projection with motion. No definite lesion identified. BOTTOM RIGHT—Right lateral projection with respiration suspended. A lesion was strongly suspected on the anterior border (arrow). At surgery, for colon carcinoma, a small metastasis (about 2 cm in diameter) was demonstrated in the location shown on the lateral scintiphotograph. In addition, a few scattered 1 cm metastases were present which could not be seen on the isotope examination. This case demonstrates the value of using multiple views as well as controlling respiration. Both of these are possible by combining the high count rates available from ^{99m}Tc with the sensitivity of the gamma camera.

tation of the motion of the left diaphragm, thus further obscuring the left hepatic lobe and spleen. In addition, it is certainly a more cumbersome technique than simple breath holding.

In the future it may be possible to exploit digital scanning readout systems by feeding them to a computer programmed to correct for respiratory motion, and it is conceivable that a scanner could be developed that would oscillate with respiration during the scan pass.

In the present day practice of nuclear medicine, undue emphasis has been placed upon the desirability of having a single imaging machine that will scan anything, and there is widespread controversy concerning the relative values of scanners and cameras, different types of cameras, or different scanners. At the University of Chicago we believe that it is better to adapt the equip-

ment to perform a limited role. For example, all brain scans are done on the high speed multi-headed Argonne Cancer Research Hospital Brain Scanner with special isoresponse collimators designed for ^{99m}Tc.[10] On the other hand, when time is of less importance and the highest obtainable resolution is desirable, as in thyroid scanning, a slow scan speed, low energy ^{125}I, and very fine focussed Beck designed collimators are used.[11] In keeping with this concept, when count rates are high enough to make this technique feasible, we prefer to study all moving organs with the gamma scintillation camera and breath holding.

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ESTROGEN PHARMACOLOGY. III. THE EFFECT OF ESTRADIOL ON THE PLASMA DISAPPEARANCE RATE OF BSP IN MAN*

By

L. L. Kottra,† and A. Kappas

Estradiol depresses the hepatic secretory transport maximum (T_m),[1,2] for sulfobromophthalein (BSP) in man.[3] In rats, this hormone similarly delays hepatic removal from plasma of both BSP and the dye phenol-3,6-dibromophthalein disulfonate,[4] a chemical analog of BSP which does not undergo conjugation during hepatic transit into bile.[5] The latter finding indicated that, in this experimental context, estrogens act on hepatic processes other than those concerned with dye conjugation and implied that the BSP retention induced by these hormones in man reflected a decrease in the net rate of hepatic disposal not only of the conjugated form of BSP, but of the free dye as well. The present study was undertaken to examine this possibility by analysis of the BSP components in the plasma of estrogen-treated subjects at appropriate intervals after intravenous injection of the dye. The results confirm that in man the net movement of both free, and conjugated, BSP out of the bloodstream is significantly decreased by estrogen treatment.

METHODS

Nine women and one man, ranging in age from 21 to 81 years, were studied. Clinical diagnosis varied; all patients were hospitalized but ambulatory during the study. Crystalline estradiol (1,3,5[10]-estratriene-3,17β-diol) was prepared in an N,N'DMA-propylene glycol solvent vehicle[3] and administered by intramuscular injection to each subject for the periods indicated in Table 1. The plasma disappearance of BSP during a control period, and on the last day of estradiol administration, was studied in each subject following a single intravenous injection of 5 mg of dye per kg of body weight; plasma BSP concentration was estimated generally at 2, 5, 10, 15, 20, 30, and 45 minutes after injection by the usual means (measurement of O.D. at 580 mμ, in an alkaline buffer). BSP components in plasma at the 20- and 45-minute intervals during the estrogen period dye disappearance curve in each subject were examined chromatographically as described previously.[3,6-8] The several known BSP conjugates in plasma were eluted, and quantitated as a single "conjugate" fraction.

Table 1 shows for each subject: the total plasma BSP concentration, 5, 20, and 45 minutes after dye injection in control and estrogen periods; the amount in milligrams per cent of the plasma BSP which was free, or conjugated, at the 20- and 45-minute intervals in the estrogen period; and the percentage decline in total plasma BSP during the 5-20 minute and 20-45 minute time intervals in the control and estrogen periods.

Figure 1 shows representative plasma disappearance curves of BSP in two subjects during control and estrogen periods. In addition, the curve for the plasma disappearance of the free

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†Formerly Student Trainee under United States Public Health Service Grant No. T1 AM 5445-01.

Table 1

PLASMA CONCENTRATIONS OF BSP DURING CONTROL AND ESTROGEN TREATMENT PERIODS IN 10 SUBJECTS

Patient—treatment	Period	Time - 5' plasma BSP mg %	Time - 20' plasma BSP - mg %			Time - 45' plasma BSP - mg %			Per cent decline in total BSP	
			Total	Free	Conjugated	Total	Free	Conjugated	5-20' interval	20-45' interval
F1 - f - Estradiol 20 mg/day x 5	Control	7.2	3.7	-	-	0.2	-	-	49	95
	Estrogen	7.7	3.4	2.7	0.7	1.8	1.4	0.4	56	47
F2 - f - Estradiol 20 mg/day x 5	Control	10.0	2.0	-	-	0.8	-	-	80	60
	Estrogen	9.9	1.6	1.1	0.5	1.3	0.8	0.5	84	19
F3 - f - Estradiol 20 mg/day x 5	Control	15.6	3.2	-	-	1.4	-	-	79	56
	Estrogen	14.6	5.2	4.1	1.1	3.3	1.9	1.4	64	37
F4 - f - Estradiol 20 mg/day x 7	Control	12.0	1.8	-	-	0.1	-	-	85	94
	Estrogen	13.0	3.3	2.7	0.6	2.4	1.3	1.1	75	27
F5 - f - Estradiol 20 mg/day x 5	Control	18.3	4.3	-	-	0.9	-	-	77	79
	Estrogen	13.2	4.5	4.2	0.3	1.4	1.1	0.3	66	69
M1 - m - Estradiol 20 mg/day x 6	Control	7.0	1.8	-	-	0.6	-	-	74	67
	Estrogen	7.4	3.6	2.8	0.8	2.7	1.4	1.3	51	25
F6 - f - Estradiol 20 mg/day x 4	Control	12.6	4.0	-	-	0.8	-	-	68	80
	Estrogen	12.6	5.0	3.8	1.2	2.7	1.5	1.2	60	46
F7 - f - Estradiol 20 mg/day x 6	Control	11.1	3.0	-	-	0.6	-	-	73	80
	Estrogen	12.0	3.5	3.1	0.4	3.0	1.6	1.4	71	14
F8 - f - Estradiol 20 mg/day x 6	Control	10.2	1.4	-	-	0.4	-	-	86	71
	Estrogen	11.0	2.4	2.1	0.3	1.2	0.6	0.6	78	50
F9 - f - Estradiol 20 mg/day x 6	Control	12.0	1.9	-	-	0.6	-	-	84	68
	Estrogen	18.0	5.9	4.9	0.6	3.0	2.4	0.6	67	49

f - indicates female.
m - indicates male.

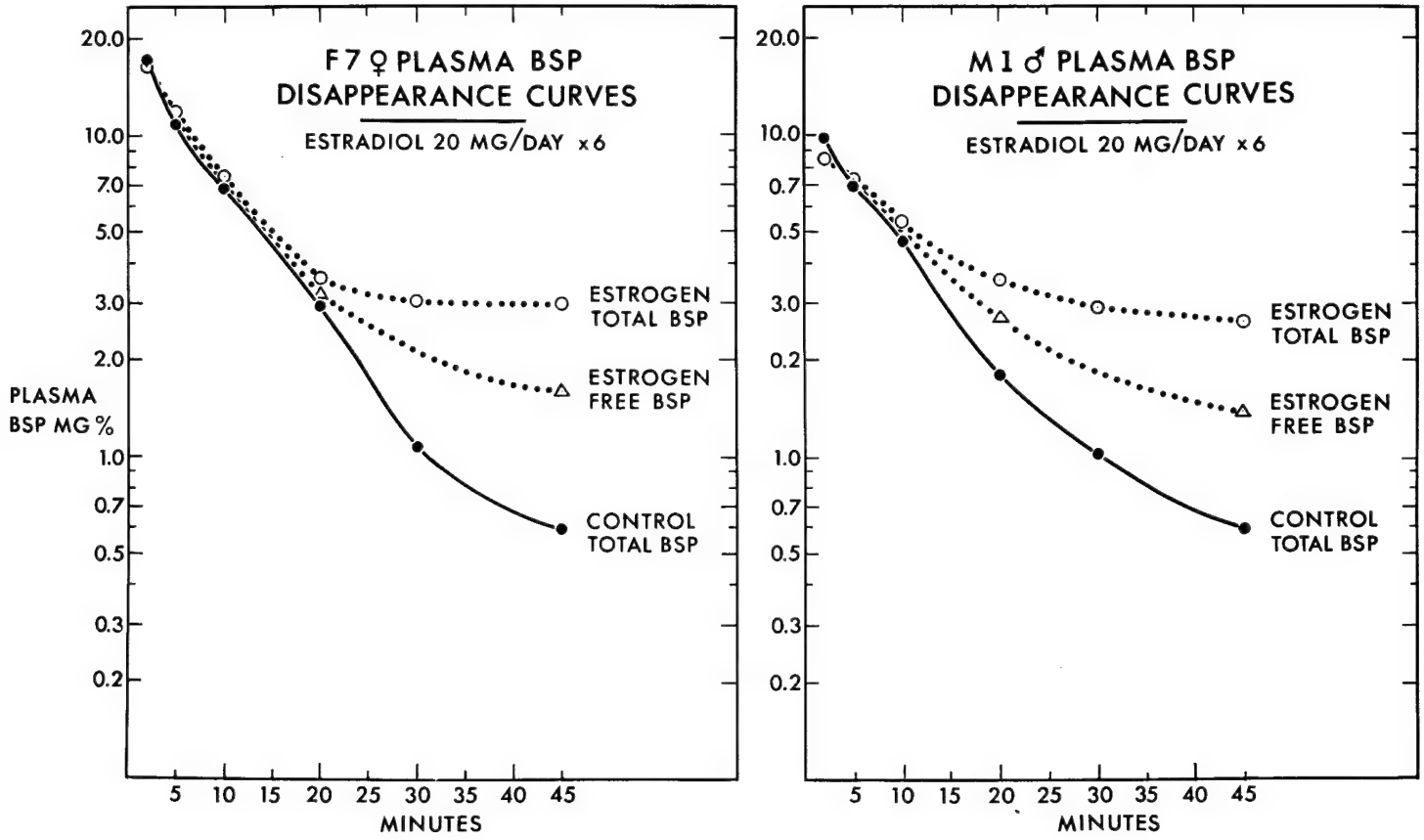

Figure 1. Representative plasma BSP disappearance curves in two subjects receiving estradiol for six days. The estrogen "free BSP" curve is based on chromatographic analysis of plasma BSP components at 20' and 45' in the estrogen period.

fraction of the total plasma BSP during the estrogen period is indicated; the plasma free BSP at the starting time (2') is presumed to equal the total plasma BSP concentration at that time.

COMMENT

Estradiol delayed plasma disappearance of BSP in each of the ten patients studied, as expected. This hormonal effect is depicted graphically in the representative dye disappearance curves in Figure 1 and reflected in the elevated 45-minute total plasma dye concentrations shown in Table 1. It is probable that nearly complete intravascular mixing of dye has occurred at the 5-minute interval after injection; it is thus of interest that there was no consistent difference in the plasma BSP concentrations at this time between control and estrogen periods. In the 5-20 minute interval a slower rate of decline in plasma BSP during the estrogen as compared with the control period was noted in several patients, and this difference became pronounced during the 20-45 minute time interval in nearly all subjects. Plasma BSP components at 20 minutes in the estrogen period showed as expected that most of the circulating dye was unconjugated; in six subjects the concentration of free dye exceeded the concentration of total plasma BSP in the control period. At 45 minutes, the absolute amount of free dye in plasma during the estrogen period equaled or exceeded the total control plasma BSP concentration at that time in all ten subjects and in eight of the ten the absolute amount of plasma conjugated BSP equaled or exceeded this value as well. These findings indicate that the impairment in BSP metabolism induced by estrogens is reflected in a delay in the net movement of both free as well as conjugated BSP out of the bloodstream into the bile; and that with respect to the characteristic shape of the normal dye disappearance curve, the effect of these hormones is manifest particularly in the latter or "excretory" portion of the curve. The resultant dye curves thus resemble those seen in neonatal children[9] and in patients with congenital excretory defects of the liver, such as the Dubin-Johnson syndrome.[10]

The high plasma concentrations of conjugated BSP during estrogen treatment shown in this and other studies,[3,4] attest to the diversion back into the bloodstream of quantities of BSP which would ordinarily be stored transiently in hepatic cells or secreted into the bile. This reflux of dye under the influence of estrogens appears to be an exaggeration of an otherwise normal, although quantitatively minor, process;[11-15] and it is not clear whether estrogens affect this process by directly enhancing the back diffusion of these conjugates from the hepatocyte (or bile radicle) into the bloodstream; by inhibiting the rate limited mechanism which transfers BSP across the cell membrane bordering the bile canaliculus, or by acting at other sites in the liver. The high plasma levels of unconjugated BSP at 45 minutes in the estrogen periods makes it impossible to exclude an additional action of these hormones such as to impede the movement of dye from the bloodstream into hepatic cells. However, the apparent similarity in initial handling of injected dye in control and estrogen periods as reflected in the dye disappearance curves and in the early plasma BSP concentrations and the fact that the hepatic storage space for BSP may increase substantially during estradiol administration[3] suggest that if present, defective entry of dye into liver cells may not be quantitatively important. Moreover the retention of excessive amounts of free as well as conjugated BSP in plasma during estrogen treatment does not <u>necessarily</u> imply delay in the initial entry of free BSP into hepatic cells; the chromatographic data reflect only the net movement of dye components out of the plasma and it is likely that the same process which leads to reflux of conjugated BSP back into the bloodstream affects the unconju-

gated dye in the same manner. Although the nature of this process is not defined, the manner in which it is affected by estrogens raises the possibility that these hormones might also impair hepatic disposal of other substances—such as drugs—which are primarily secreted into the bile via a rate-limited transport mechanism.

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PRODUCTION OF BILE DUCT HYPERPLASIA AND GALLSTONES BY LITHOCHOLIC ACID*

By

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Lithocholic acid‡ is an important metabolite of cholesterol in man and other animals. Interest in its biological properties stems from its marked toxicity. It is the most potent of the naturally occurring steroids that produce intense fever and inflammation in man[2,3] and inflammation in a number of other species.[3,4] It is also one of the most active steroid hemolysins.[5] In addition, its oral administration produces cirrhosis of the liver in rabbits[6] and ductular cell hyperplasia in a variety of species, including rodents,[7] reptiles,[8] and primates.[9] In view of its physiological occurrence, its known toxic properties, and its potential relevance to human disease, we have investigated the effects of feeding large amounts of lithocholic acid to rats. The resulting bile duct hyperplasia and choledocholithiasis are described in this report.

MATERIALS AND METHODS

The acute effects of lithocholic acid and sodium lithocholate on Sprague-Dawley rats were investigated in Experiments I and II, and chronic effects in Experiments III and IV. Lithocholic acid was obtained commercially;§ no quantitatively significant bile acid contaminants were observed when it was analyzed by thin-layer chromatography (system S15 of Eneroth[10]). The sodium salt was prepared by neutralization of the acid with sodium hydroxide.

In the first experiment, 5 male rats, average weight 372 g, were force-fed 300 mg sodium lithocholate per kg body weight per day in a liquid diet[11] containing 3.6 g casein hydrolysate per kg body weight per day. The rats were fed 3 times daily at 8-hour intervals, and killed with ether on the sixth day.

In the second experiment, 9 males and 9 females, average weight 171 g, were divided into 3 equal groups and force-fed the basic liquid diet as described above. One group served as controls. A second group was fed lithocholic acid, 300 mg per kg body weight per day, in the diet. The third group received sodium lithocholate, 300 mg per kg body weight per day, in the diet. All rats were killed with ether on the twelfth day.

In the third experiment, 10 male and 10 female control rats (average weights: males, 193 g; females, 157 g), were fed ad libitum an 8 per cent protein diet,¶ containing 8 per cent casein,

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‡Trivial names of bile acids used in this report are: lithocholic acid, 3α-hydroxy-5β-cholanoic acid; 6β-lithocholic acid, 3α,6β-dihydroxy-5β-cholanoic acid; hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid.

§Nutritional Biochemicals Corp., Cleveland, Ohio.

¶"Low 8% Protein," Nutritional Biochemicals Corp., Cleveland, Ohio.

78 per cent starch, 10 per cent vegetable oil, and 4 per cent salt mixture U.S.P. XIV, to which had been added 1 kg "Vitamin Diet Fortification Mixture"* per 100 lbs diet. A similar group of rats (average weights: males, 193 g; females, 157 g) was fed the same diet containing 1 per cent lithocholic acid. The powdered diets were mixed with sufficient water to permit molding into conveniently sized balls. Generally a 1 to 2 weeks' supply was made up and refrigerated. A ball was placed in each cage every 2 to 4 days, depending on spillage. At the end of the fourth month the surviving animals were killed by decapitation.

In the fourth experiment, 5 control and 15 treated rats (150 to 200 g) were force-fed the liquid diet of the first experiment for 1 month and then placed on the solid diet of the third experiment ad libitum. The treated animals received sodium lithocholate, 300 mg per kg per day in the liquid diet and 1 per cent in the solid diet. After 4 months, the surviving animals were killed.

At autopsy, livers were weighed and tissue samples fixed in buffered formalin and Carnoy's fluid. Sections were stained with hematoxylin-eosin, Mallory trichrome, PAS, and methyl green-pyronine.

For electron microscopic studies, rat liver samples from Experiment I were fixed for 1 hour in 1 per cent osmic acid buffered with s-collidine at pH 7.4, dehydrated in graded alcohols and embedded in methacrylate. Three extrahepatic duct calculi from Experiment III were fixed in 2 per cent osmic acid for 24 hours, dehydrated in absolute alcohol for 12 hours, and embedded in methacrylate. The sections were stained with lead hydroxide.[12]

Gallstone analysis. Gallstones were stored in the freezer under nitrogen prior to analysis. Stones from individual animals were crushed and weighed. Aliquots of 25 mg were dried at 110° C for 24 hours to obtain the per cent dry weight, and then ashed for mineral analysis. Calcium was measured with a Perkins-Elmer atomic absorption spectrophotometer, and sodium and potassium by flame spectrophotometry. A second aliquot of 100 mg of crushed stones was homogenized, in a Waring blender, in 25 ml $CHCl_3$:MeOH, 1:4, and portions of the suspension were used for bile acid, total lipid,[13] and bilirubin[14] determinations. Cholesterol, in a third aliquot of 20 mg, was measured by the Tschugaeff reaction.[15]

Qualitative bile acid analysis was performed by thin-layer chromatography, with the solvent systems described by Gänshirt, Koss, and Morianz[16] and Eneroth,[10] both before and after the sample was hydrolyzed in 5 N NaOH for 24 hours at 130° C. Total bile acids were quantitated enzymatically using 3α- and 3β-hydroxy-steroid dehydrogenases. The method (to be reported in detail elsewhere) is similar to that used by Hurlock and Talalay[17] for the analysis of neutral steroids, and applied by Iwata and Yamasaki[18] to bile acids.

Isotopic studies. Sodium lithocholate-24-^{14}C, obtained from Nichem, Inc., Bethesda, Maryland, had a specific activity of 1 mc/mg, and was prepared according to the method of Bergstrom, Rottenberg, and Voltz.[19] Animals were housed in individual metabolism cages, and urine and feces were collected separately. After an intraperitoneal injection of approximately 4×10^6 DPM of sodium lithocholate-24-^{14}C in 0.5 ml of 50 per cent ethanol, aliquots of urine and of $CHCl_3$:MeOH extracts of feces and intestinal contents were counted in POPOP using a Nuclear-Chicago liquid scintillation counter with internal quench correction. Labeled bile acids in intestinal contents were chromatographed in a solvent system consisting of butanol 50 ml, acetic acid 5 ml,

*"Low 8% Protein," Nutritional Biochemicals Corp., Cleveland, Ohio.

and water 5 ml, as described above, and radioactivity was detected with a Vanguard "885" glass plate scanner. Standard spots of glycocholic and taurolithocholic acids were located by spraying with phosphomolybdic acid.[20] The per cent of labeled compounds conjugated with taurine was calculated by cutting out and weighing the areas under the appropriate curves on the recorder strip.

Miscellaneous. Urinary taurine was measured by the method of Bergerat and Chatagner,[21] except that the ninhydrin reaction was performed according to Rubinstein and Pryce.[22] Serum cholesterol was determined by Sackett's modification of Bloor's method.[23]

RESULTS

Morphological changes. In the acute experiments, the rats appeared to be normal and no gross pathological changes were noted at autopsy except that one rat in the sodium lithocholate group of Experiment II had a small soft calculus in the distal hepatic duct and some dilatation of the duct proximally. In the chronic experiments, the treated rats lost hair and often assumed a hunched posture. At the time of autopsy, as shown in Table 1, treated female rats had not gained

Table 1

BODY AND LIVER WEIGHTS OF RATS FED LITHOCHOLIC ACID FOR FOUR MONTHS
(Experiment III)

	No. surviving	Initial weight	Average weight gain ± SD	p value	Average liver weight ± SD	p value
		g	g		g	
Female controls	10	157	70.4 ± 12.6	< 0.05	6.00 ± 0.71	< 0.025
Female treated	8	157	55.0 ± 13.2		7.16 ± 1.13	
Male controls	10	193	96.9 ± 13.8	*	9.18 ± 1.70	< 0.01
Male treated	7	193	100.1 ± 21.7		11.87 ± 1.67	

*Not significant.

weight as well as controls, but treated male rats had gained normally. Liver weights in treated animals of both sexes were significantly increased over controls. In both chronic experiments, however, the most striking pathological feature was the presence of large common duct calculi in 100 per cent of the treated rats (Figures 1 and 2). The stones were usually multiple, yellow or green, and soft and friable, but marked variations in color, size, and shape were apparent.

Livers of rats fed lithocholic acid or sodium lithocholate in both acute and chronic experiments, showed microscopic changes that were essentially similar, and limited to the biliary system. The portal areas of the liver showed marked bile duct proliferation and variable mononuclear cell infiltration (Figure 3). Within bile ducts, basophilic crystalline deposits (Figure 4) with staining characteristics similar to those of lithocholic acid embedded in agar (Figure 5) were frequently observed. Ductal cells, some undergoing necrosis, were seen associated with eosinophilic debris in the lumens of the bile ducts (Figure 6). A similar eosinophilic material often surrounded the basophilic crystalline deposits, either loosely (Figure 7) or more densely (Figures 8 and 9) to form micro-calculi. Dense infiltrates of eosinophilic leukocytes were seen

Figure 1. Biliary tract of rat fed sodium lithocholate for 4 months (Experiment IV). The enlarged common bile duct (arrow) is shown with adherent fat. Liver (L), intestine (I). X 2.6.

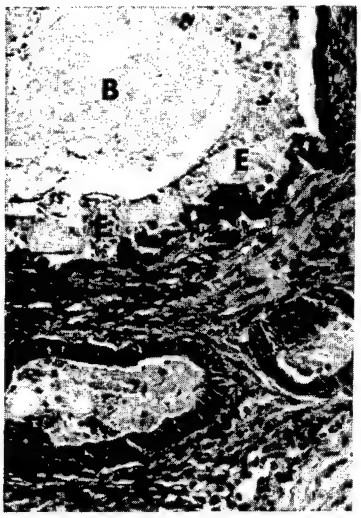
Figure 2. Rat shown in Figure 1 after opening the common duct. Calculi of various sizes are shown.

Figure 3. Portal area of liver from a rat fed lithocholic acid for 4 months (Experiment III). Bile duct proliferation and pericholangitis are present. Some ducts are distended and contain amorphous material. H and E stain. X 102.

Figure 4. Crystalline basophilic material from the center of a calculus.

Figure 5. Lithocholic acid in agar gel. Figures 4 and 5, H and E stain. X 1,440.

Figure 6. Intrahepatic bile ducts from a rat fed sodium lithocholate for 4 months (Experiment IV). Basophilic crystalline material is seen on the right, the wall of the duct with desquamating cells is on the left. H and E stain. X 540.



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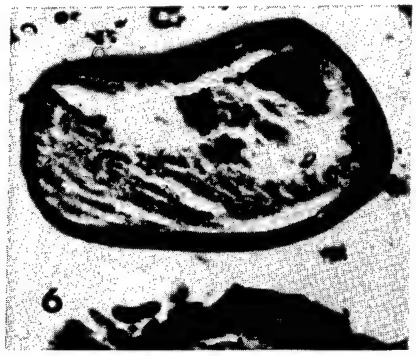

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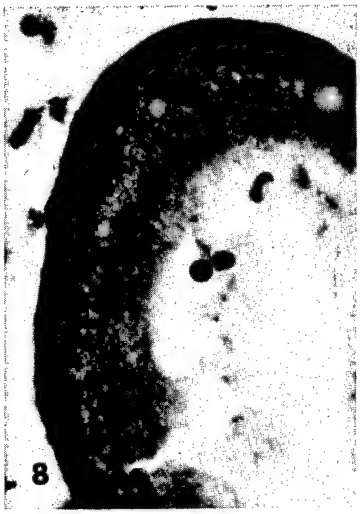

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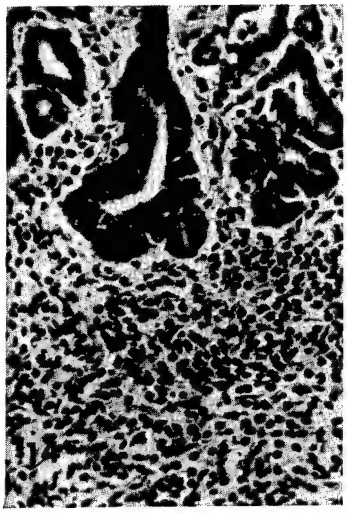

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Figure 7. Liver section as in Figure 6. The large duct contains basophilic material (B) centrally and eosinophilic material (E) peripherally. H and E stain. X 222.

Figure 8. Calculus in a large intrahepatic bile duct from a rat fed sodium lithocholate for 4 months (Experiment IV). The center contains basophilic, densely clumped crystalline material; the capsule is eosinophilic. H and E stain. X 558.

Figure 9. Calculus as in Figure 8. The densely packed outer shell and the reticular inner layer are discernable. H and E stain. X 1,440.

Figure 10. Portion of the wall of the common duct from a rat fed lithocholic acid for 4 months (Experiment III). Polymorphonuclear cells, predominantly eosinophiles, infiltrate the wall. H and E stain. X 240.

around damaged bile ducts, and in the wall of the extremely hypertrophied common duct (Figure 10). Although focal hepatic necroses were found occasionally, the hep ocytes were generally intact, with normal glycogen preservation and the usual cytoplasmic basophilic bodies. Ultra-

structural studies of rat livers from the first experiment showed only hyperplasia of the Golgi complex and an increase in the amount of smooth endoplasmic reticulum; the bile canaliculi were not distended. Electron micrographs of the calculi revealed electron dense flaky material in a lucid matrix, similar but not identical to the appearance of calcium lithocholate prepared similarly.

Composition of lithocholic acid-induced rat gallstones. The calculi in Experiment III had average dry weights of 5.97 ± 0.77 g for males and 4.14 ± 1.75 g for females. Gallstones from 15 of the rats were analyzed, with the results given in Table 2. Eight-five per cent of the dry weight was accounted for by the analyzed constituents, primarily bile acids. Small amounts of non-diazo reacting pigments, carbohydrate, and probably protein and nucleic acids were also present, but in the presence of large amounts of bile acids, identification was not reliable.

Table 2

COMPOSITION OF LITHOCHOLIC ACID-INDUCED GALLSTONES

	Females	Males
Wet weight ± standard deviation (g)	5.04 ± 1.68*	7.39 ± 0.24*
Per cent water (range)	19.1(5.5-54.6)	21.8(9.4-34.8)
Dry weight ± standard deviation (g)	4.14 ± 1.75†	5.97 ± 0.71†
Bile acids (calculated)	71%	69%
Ash	12%	12%
(Ca)	(3.3)	(3.0)
(Na)	(.77)	(.81)
(Mg)	(.16)	(.15)
(K)	(.07)	(.08)
(P)	(.02)	(.02)
Total lipid	0.9%	0.4%
(Cholesterol)	(.04)	(.04)
Bilirubin	.12%	.17%

*$p < 0.005$.
†$p < 0.05$.

The relative amounts of bile acids and cations indicated that the bile acids occurred primarily as calcium salts. The composition in mEq per g dry weight was (mean ± standard deviation): bile acids, 1.57 ± 0.24; Ca, 1.59 ± 0.23; Na, 0.32 ± 0.18; K, 0.19 ± 0.12; Mg, 0.13 ± 0.02.

Analysis of the bile acids in several stones by thin-layer chromatography showed varying proportions of free and glycine-conjugated compounds; only traces of taurine-conjugated bile acids were present. Thin-layer chromatography of the bile acid extract after alkaline hydrolysis showed a consistent pattern from animal to animal; the predominant steroids were lithocholic and 3α,6β-dihydroxycholanic acids, with smaller amounts of hyodeoxycholic and cholic acids and traces of chenodeoxycholic acid being detected. Quantitative fractionation of the bile acids in hydrolyzed extracts of gallstones from four representative animals is shown in Table 3. Between 93 and 100 per cent of the bile acids present in the extract were recovered by eluting the

Table 3

ANALYSIS OF BILE ACIDS IN HYDROLYZED GALLSTONE EXTRACTS

	1		2		3		4	
	mμmoles	%	mμmoles	%	mμmoles	%	mμmoles	%
Total bile acids	44.5	100.0	41.4	100.0	43.8	100.0	40.9	100.0
Lithocholic acid	12.2 13.3 13.6	29.1	15.7 15.9	38.6	12.3 10.7 11.3	26.3	12.3 11.6	29.0
6β-hydroxylitho-cholic acid	19.0 18.8	42.4	10.9 11.5	27.1	18.3 19.7 18.6	42.9	15.7 14.2 15.4	36.4
Hyodeoxycholic acid	4.2 4.1	9.2	5.8 5.9	14.3	4.9 3.5 6.1	11.0	7.8 7.8	18.8
Cholic acid	3.6 5.2 7.0	11.9	6.5 4.5 6.4	14.0	5.4 4.9	11.6	4.6 6.1 6.4	13.8
Chenodeoxycholic acid	0	0	1.5 2.8 2.9	5.8	0	0	0	0
Total		92.6		99.8		91.8		98.0

A 5 μl aliquot of the hydrolyzed chloroform:methanol gallstone extract from each of 4 animals was assayed directly to give the total bile acid value, designated 100%. Three other 5 μl portions were chromatographed using phase system S11 (10). Individual spots were eluted and assayed. The average value from 2 or 3 chromatographies was used to determine the fractional bile acid composition.

component spots, and there was good agreement between replicate chromatographies. No bile acids with 3β-hydroxyl groups were detected.

Prevention of stone formation. Because of the remarkable paucity of taurine conjugates in a species normally conjugating bile acids predominantly with taurine, we investigated the effects of protein or taurine dietary supplements on stone formation. Four groups of 5 males and 5 female rats were maintained on the diets listed in Table 4 for 8 weeks. The basic diet was the same as that used in Experiment III. As expected from the results of Experiment III, none of the 8 per cent protein control rats (Group I) and all of the 8 per cent protein, lithocholic acid-treated rats (Group II) developed large accumulations of common duct stones (average weight 4.15 g). In contrast, in the rats receiving the protein-supplemented lithocholic acid diet (Group III), there were only 2 females with a few very small stones. In rats receiving the taurine-supplemented lithocholic acid diet (Group IV), there were no stones. Some rats in Groups III and IV, usually females, had thickened and slightly dilated common bile ducts, though no obstruction was apparent. The bile was generally thick and some precipitated sludge was observed. The extrahepatic ducts appeared to be normal in the remaining animals. Protein supplementation resulted in greatly increased growth, whereas taurine supplementation did not.

In order to investigate the metabolic changes associated with dietary prevention of stone formation, several animals in each group were studied prior to autopsy. Urinary taurine excre-

Table 4

MODIFICATION OF THE EFFECTS OF EIGHT WEEKS TREATMENT WITH LITHOCHOLIC ACID BY DIETARY SUPPLEMENTS

Group	Diet	Average weight gain	S-containing amino acids in the diet	Urinary taurine
		g	μmoles/g diet	μmoles/24 hours*
I	8% protein	44	14.3	15.9 ± 0.3
II	8% protein 1% lithocholic acid	30	14.3	17.6 ± 2.2
III	27% protein 1% lithocholic acid	111	47.9	31.0 ± 8.4
IV	8% protein 1% lithocholic acid 1% taurine	40	94.1	70.0 ± 13.8

*Mean ± standard deviation.

tion was determined in 2 males and 2 females in each group, and the results are shown in Table 4. The urinary taurine closely reflected the total sulfur-containing amino acid content (including taurine) of the diet.

Two different males and females in each group were given intraperitoneal injections of sodium lithocholate-24-^{14}C (4 x 10^6 DPM), 3 days prior to autopsy. Urinary bile acid-^{14}C excretion was determined, and at autopsy the small intestinal bile acid pool was analyzed for taurine-conjugated labeled bile acids. The results are shown in Table 5. Urinary excretion of labeled bile acids was similar in all groups, but females excreted more isotope in the urine on day 1 than did males ($p < 0.01$).

Analysis of labeled bile acids in the intestinal contents revealed significant enhancement of taurine conjugation in Groups III and IV ($p < 0.01$). The proportion of bile acids conjugated with taurine rose with increasing amounts of sulfur-containing amino acids in the diet, and correlated well with the observed inhibition of stone formation.

Effects of diet on bile duct hyperplasia. Histological examination of the livers revealed no significant differences between the extent of bile duct proliferation in Groups II, III, and IV, although the proliferation tended to be slightly more intense in Group II. No correlation could be made with stone formation.

DISCUSSION

The production of gallstones in any species by orally administered lithocholic acid has not been reported previously. Rat gallstones are extremely uncommon, presumably due to the absence of a gallbladder in this species, and the usual methods of producing gallstones experimentally in other species have not been effective in rats. The consistent production of common duct gallstones in rats by lithocholic acid, as described in this report, represents an important new toxic effect of this steroid, and provides a new experimental model for the study of gallstone formation. In addition, the direct participation of one of the bile acids endogenous to man and

Table 5

EFFECTS OF DIETARY ALTERATIONS ON THE METABOLISM OF LITHOCHOLIC ACID-24-^{14}C INJECTED INTRAPERITONEALLY*

Group	Animal	Sex	Isotope recovered in urine - day 1	Labeled bile acids recovered in intestinal contents at autopsy	
			DPM x 10^{-4}	Per cent conjugated with taurine	Mean ± standard deviation
I	1	F	13.8	0	
	2	F	10.5	21	
	3	M	7.6	17	
	4	M	6.5	13	
					13 ± 7.8
II	5	F	9.3	7	
	6	F	10.7	22	
	7	M	6.9	9	
	8	M	8.1	6†	
					11 ± 6.6
III	9	F	6.8	42	
	10	F	8.9	57	
	11	M	5.2	17	
	12	M	9.2	85	
					50 ± 25
IV	13	F	7.3	48	
	14	F	13.9	67	
	15	M	4.0	40‡	
	16	M	5.2	96	
					63 ± 22

*4×10^6 DPM injected.

†13% free bile acids.

‡8% free bile acids.

other species in the production of biliary calculi has important implications for current concepts of the pathogenesis of cholelithiasis.

The pathogenesis of the gallstones induced in the present studies can be inferred from histological and chemical data. An early step in stone formation appears to be the precipitation of the calcium salts of free and glycine-conjugated lithocholic acid and its 6β-hydroxy-derivative in the small bile ducts. These steroids are less soluble than most common bile acids, and since calcium salts of bile acids are less soluble than sodium salts, the precipitation of these particular compounds is not surprising. In rabbits fed cholestanol, an analogous precipitation of calcium glycoallodeoxycholate also leads to stone formation.[24,25] The composition of the stones resembles that of the naturally occurring pig gallstones, which consist mainly of lithocholic and 3β,6α-dihydroxy-5β-cholanic acids.[26] Cellular debris, resulting from the intense desquamative and cytotoxic effects of lithocholic acid, apparently facilitates stone formation by helping to bind precipitated bile salts into microcalculi (Figures 6-8). Stones formed in this manner probably enlarge by accretion, and any stasis developing secondary to partial obstruction would undoubtedly enhance this process. Finally, microbial modification of biliary constituents may be reflected in the stone composition. Variations in the occurrence, type, or extent of biliary infection in these

experiments probably accounted for the different pigments and proportions of free and conjugated bile acids formed and incorporated into these stones. Similarly, through effects on local pH and on conjugate hydrolysis, bacterial infection may have promoted further stone formation.

The prevention of lithocholic acid-induced gallstones by dietary supplements of protein or taurine is an interesting finding. Taurine is normally used preferentially for bile acid conjugation by the rat,[27] and the supply of taurine is known to be rate-limiting in this reaction.[28] The low level of taurine conjugation in rats receiving the 8 per cent protein diet (Groups I and II) suggests that this diet is deficient in the sulfur-containing amino acids (cystine and methionine) which are prerequisites for taurine formation. A deficiency of this type in the 8 per cent protein diet is also suggested by the fact that rats gained much more weight on the 27 per cent protein supplemented diet (Group III) than on the 8 per cent protein diet supplemented with taurine (Group IV), and it has been shown previously that taurine cannot replace the growth requirement for cystine and methionine.[29] Therefore, the ability of lithocholic acid to induce stone formation in animals on the low protein diet is probably related to the lack of sufficient sulfur-containing amino acids in this diet. Increasing the supply of actual or potential dietary taurine leads to increased availability (Table 4) and increased utilization of this amino acid for bile acid conjugation (Table 5), and results in a marked decrease in stone formation. It would be of interest to know whether taurine conjugation of allodeoxycholic acid could prevent cholestanol-induced gallstones in rabbits, and whether taurine administration or a high protein diet might be of benefit in some cases of human cholelithiasis.

These studies, demonstrating that lithocholic acid-induced cholelithiasis can be inhibited by enhancing bile acid conjugation with taurine but not glycine, again emphasize the importance of the specific conjugating substance in determining the physiological or pathological activity of bile acids. The exact mechanism by which taurine conjugation inhibits stone formation is not clear, but presumably the strongly polar sulfate group of taurine results in a more polar bile acid conjugate with a low pK. This conjugate, while still relatively insoluble, is considerably more soluble than glycine-conjugated or free lithocholate. We have previously reported that the pyrogenic activity of lithocholic acid in man is abolished by conjugation of the steroid with taurine but not with glycine.[3] Isselbacher and his co-workers have described differences between the effects of taurine and glycine conjugated bile salts on glucose and fat metabolism in the small intestinal mucosa.[30,31] Other substances, such as sulfate[26] and ornithine,[32] which may be found conjugated with bile acids might also therefore be expected to affect their physiological or pathological properties. Ornithine-conjugated bile acids, with polarity characteristics intermediate between those of taurine and glycine conjugated bile acids, have been described by Peric-Golia and Jones[33] in the bile of certain patients with cholelithiasis. In man, lithocholic acid occurs to a significant extent as a conjugated compound more polar than either taurolithocholic or glycocholic acids.[34] The nature of this conjugating substance is unknown, but in view of the toxic effects of lithocholic acid, investigations on the composition and properties of this conjugate will be of considerable interest.

The urinary excretion of ^{14}C-labeled bile acids was similar in animals on different diets, with or without stones, but females in all groups consistently excreted significantly more isotope than did males. This sex difference could result from a difference in the bile acid pool sizes, from a difference in the renal tubular reabsorption of bile acids,[35] or from an inhibitory effect of estrogens on biliary bile acid excretion analogous to their effect on BSP excretion, which has

been studied extensively by Kappas and collaborators.[36,37] A sex difference in ability to excrete toxic bile acids into the bile might be of importance in considering the increased severity of liver disease in females, particularly during pregnancy.

In addition to gallstone formation, a second and equally important effect of lithocholic acid in these experiments was the production of marked bile duct proliferation and hyperplasia of the common duct mucosa. Liver damage induced by lithocholic acid was first observed by Holsti in 1956.[6] He described cirrhosis of the liver in rabbits fed whole or dessicated hog bile,[38] and in a series of experiments, showed that this cirrhosis could be produced by a bile acid extract of hog bile,[39] by lithocholic acid,[6] and by glycolithocholic or chenodeoxycholic acids.[40] Stolk has confirmed this effect of lithocholic acid in reptiles.[8] Bile ductal and ductular cell hyperplasia in chickens fed lithocholic acid has also been reported by Hunt, Leveille, and Sauberlich.[41] Eyssen, Vandeputte, and Evrard[42] found that lithocholic acid, taurolithocholic acid, and 3-ketocholanic acids could all produce these changes in chickens, but that chenodeoxycholic acid could not. Similar changes have been observed in guinea pigs, hamsters, and monkeys,[7,9] and to a lesser extent in rats and mice.[7,42] The apparent resistance of rats to these effects of lithocholic acid is of interest, and could have been related in part to the presence of enzyme systems in rat and mouse livers capable of catalyzing the extensive hydroxylation of lithocholic acid at the 6α, 6β, and 7α positions[43] and thus presumably inactivating it. Such changes have been shown to decrease the hemolytic and cytotoxic activity of lithocholic acid.[3-5] Therefore, large doses of lithocholic acid were used in these experiments and extensive bile duct proliferation was noted in all animals, both on the low (8 per cent) and normal (27 per cent) protein diets.

The pathological changes produced by lithocholic acid in rats were slightly different from those seen in chickens and rabbits. Marked bile duct proliferation was present, but sheet-like or finger-like areas of proliferating ductular cells, the "ductular cell reation"[41] seen in other species, were not as prominent. Instead, large numbers of infiltrating eosinophilic leukocytes were seen in portal areas with damaged bile ducts, and in the walls of the large hepatic and common bile ducts. These cells have not been described as part of the response to lithocholic acid in other species, and their significance is entirely speculative.

The ductular reaction apparently occurred without significant mechanical obstruction. It was seen in acute experiments without demonstrable extrahepatic stone formation (increased incorporation of tritiated thymidine occurs within 24 hours[44]); there was extensive epithelial proliferation in the common duct down to the point where it entered the duodenum (presumably below any obstruction); and electron micrographs failed to show canalicular dilatation. The relation between biliary tract obstruction and cellular proliferation was investigated by Jacoby in the guinea pig.[45] Ligation of the common duct produced an increase in mitotic activity of all cells in the gallbladder, starting with the epithelial cells. Ligation of the cystic duct and distention of the gallbladder with paraffin did not result in increased mitotic activity, indicating that increased pressure was not the sole stimulus for proliferation. Fry and Staffeldt[46] have shown that deoxycholate-fed mice have a greatly increased cell turnover, in both the gallbladder and the small intestine, as determined by the uptake of tritiated thymidine, but ductal proliferation was not observed. These studies are consistent with the idea, suggested by Hunt and co-workers,[7] that excretion of lithocholic acid or a metabolite may be a specific stimulus to ductal or ductular cell hyperplasia. The similarity between the early and late lesions in our rats, together with the absence of cirrhosis in the chronic experiments, is consistent with the hypothesis that the lesions are directly related to the continu-

ing stimulus of some such agent. Furthermore, when lithocholic acid is removed from the diets of rabbits[38] and chickens,[41] the lesions regress. Alternatively, the proliferation may be simply a non-specific response to the cytotoxic effects of lithocholic acid. Unpublished studies indicate that cellular proliferation induced by lithocholic acid can be largely prevented by the simultaneous feeding of cholic acid and cholesterol, even in the presence of significant stone formation. In contrast, as reported here, taurine supplementation suppressed stone formation but not ductal proliferation, suggesting that ductal hyperplasia and gallstone formation occur by different mechanisms.

The liver and biliary tract changes seen in rats following lithocholic acid administration were remarkably similar to those produced by Vitamin A deficiency.[47,48] Hamre observed extensive bile duct proliferation in several of his Vitamin A deficient rats,[48] and in a high percentage of animals he found epithelial cells sloughing off in the small bile ducts and accumulating behind the ampula to form large calculi. He also noted eosinophilic infiltration in the wall of the common duct around the stones. The similarity between these findings and those in lithocholic acid-fed rats is striking; however, specific signs of Vitamin A deficiency were not observed in our rats, and further experiments have shown that Vitamin A administration does not inhibit lithocholic acid-induced stone formation despite the presence of greatly increased liver and serum levels of Vitamin A.

Lithocholic acid-induced cholelithiasis and bile duct proliferation in rats has relevance to human disease because of the important role of lithocholic acid in human bile acid metabolism. Lithocholic acid is formed in the intestine as a result of bacterial dehydroxylation of chenodeoxycholic acid at carbon 7. The transformation is an efficient one, in view of the paucity of C7 hydroxylated bile acids in feces, and a reasonable estimate for the daily production of lithocholic acid in humans would be 100 to 400 mg. In some individuals, lithocholic acid may undergo further metabolic transformations, mainly to the 3β-hydroxy-isomer, isolithocholic acid,[34] and it is now generally agreed that these two monohydroxycholanic acids form a large (25 to 50 per cent) fraction of the total fecal bile acids.[49-51] The extent of lithocholic acid metabolism, which is highly variable, may well be of great importance in determining the amount of lithocholic acid absorbed from the intestine; its major metabolite, isolithocholic acid, apparently is not well absorbed and exceedingly small amounts are present in bile.[34] Lithocholic acid is absorbed however, and has been tentatively described in human serum by Mihaesco and Fauvert[52] and Sandberg, Sjövall, Sjövall, and Turner.[53] It has been isolated and identified in normal and pathological sera by Carey,[54] and we have determined its concentration in several instances.* It is excreted in the bile, but due to technical difficulties in its estimation, there is very little reliable, quantitative data. Wootton and Wiggins[55] examined the bile from 10 patients and found that lithocholic acid comprised 5 per cent of the total bile acids in one patient; recovery data were not given for their method. Based on an estimated daily biliary excretion of 20 to 30 g of bile acids,[56] this would correspond to a daily biliary excretion of lithocholic acid in excess of 1,000 mg in that patient. (Six milligrams of lithocholate injected intramuscularly or intravenously are sufficient to produce intense fever and local inflammation in humans.[3]) Lithocholic acid has also been described in bile in similar proportions by Hauton, Greusard, Perrot, and Sarles,[57] and Kuksis,[51] but the problems of extraction, hydrolysis and recovery of lithocholic acid are such[34,54] that these fig-

*The levels of lithocholic acid in 3 jaundiced patients were 0.13, 0.45, and 0.73 μM per cent, of 0.49, 1.70, and 2.75 μg per ml.

ures may be regarded as minimal. Rosenfeld has isolated 91 mg of lithocholic acid as the methyl ester from 41 ml of human gallbladder bile,[58] a figure close to 5 per cent of the total estimated pool size. More meaningful data, with respect to gallstone formation, would relate to the concentration of lithocholic acid relative to its solubility in bile; unfortunately these are not available, but lithocholic acid and its taurine and glycine conjugates are all highly insoluble in aqueous media, even at neutral pH. Lithocholic acid has been reported among free bile acids isolated from human gallstones,[59,60] and recently it has been shown to produce cholestasis following its intravenous infusion in rats,[61] an activity of importance with respect to both bile duct proliferation and gallstone formation. The presence of this highly insoluble and inflammatory substance in human bile and gallstones strongly suggests that serious consideration be given to its possible role in human as well as animal cholelithiasis.

Finally, lithocholic acid must be regarded as an endogenous compound potentially capable of producing bile duct proliferation in human liver disease. It has the capacity to produce this lesion in all species studied to date, from reptiles to mammals, and its daily production in humans is of the same order of magnitude as that required to produce bile duct proliferation in rabbits and chickens. Particular consideration should be given to its possible role in diseases such as ulcerative colitis, where protein deficiency frequently co-exists with a loss of mucosal integrity, and where large amounts of lithocholic acid might be absorbed and presented to an unusually susceptible liver.

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RNA THIOLASE: THE ENZYMATIC TRANSFER OF SULFUR FROM CYSTEINE TO sRNA IN ESCHERICHIA COLI EXTRACTS

By

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Recently, several investigators have reported the occurrence of sulfur-containing nucleotides as minor constituents of bacterial and mammalian soluble ribonucleic acid.[1-4] Lipsett[2] has carried out an elegant characterization of 4-thiouridylic acid isolated from Escherichia coli sRNA,† and Peterkofsky and Lipsett have demonstrated that the sulfur moiety is probably derived from cysteine.[5] Because of the wide variety of unusual nucleotides present in sRNA, the biosynthesis of this important ribonucleic acid, which plays a central role in protein synthesis, offers an interesting challenge. Two basic mechanisms may be visualized for the biosynthesis of minor nucleotides in an RNA molecule: (a) insertion during nucleotide polymerization; or (b) modification of nucleotides after polymerization. The discovery by Fleissner and Borek of RNA methylase activity[6] and extension of this work by others,[7] as well as preliminary studies in this laboratory on pseudouridine formation,[8] indicate that certain nucleotides can be biochemically altered after polynucleotide assembly. We now wish to present some preliminary observations on the enzymatic transfer of sulfur from cysteine to sRNA. The enzymatic reaction is catalyzed by a soluble extract prepared from Escherichia coli lysates, and the use of periodate-treated sRNA facilitates detection of the RNA thiolase activity. A nonenzymatic reaction, distinct from the one above, is also described.

MATERIALS AND METHODS

Radioactive cystine (^{35}S-labeled, or uniformly labeled with ^{14}C) was purchased from New England Nuclear Corp.; a tenfold excess of 2-mercaptoethanol was added to reduce the cystine to cysteine, and the material was stored at -20°. E. coli DNA was prepared by the procedure of Marmur.[9] Rat liver sRNA and E. coli ribosomal RNA were prepared by phenol extraction of a rat liver 105,000 X g supernatant fraction, and isolated E. coli ribosomes, respectively. E. coli sRNA (strain K12 and B, stripped), yeast sRNA, polyadenylic, polyuridylic, and polycytidylic acids were obtained from commercial sources. Nucleic acid was treated with periodate in the following manner: A solution of RNA or DNA (3-5 mg per ml in 0.02 M Tris, pH 7.5) was adjusted to contain 0.1 M $NaIO_4$ and allowed to stand for 30 min at room temperature; periodate was then removed from the solution by exhaustive dialysis against the same buffer, at 4°. 4-thioUMP was isolated from E. coli sRNA by alkaline hydrolysis and DEAE-cellulose chromatography as described by Lipsett.[2]

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†The abbreviations used in this report are: sRNA, soluble ribonucleic acid; IO_4^--RNA, periodate treated RNA; Rb RNA, ribosomal RNA; DNA, deoxyribonucleic acid; poly U, poly A and poly C, the ribohomopolymers of uridylic, adenylic and cytidylic acids; CTAB, cetyltrimethylammonium bromide; NMP, nucleoside monophosphate; UMP, uridine monophosphate; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid.

Enzyme preparation. E. coli Q13 (a gift from Dr. S. Spiegelman), a derivative of E. coli K12 deficient in ribonuclease and RNA phosphorylase, was grown in MS broth,[10] harvested at 3/4 log phase, washed once in isotonic NaCl-phosphate buffer, pH 7.5, and stored frozen. Frozen cells were suspended at 1 g per 5 ml in a solution containing 0.02 M Tris, pH 7.5, 0.002 M $MgCl_2$, and 0.01 M 2-mercaptoethanol and subjected to sonic oscillation for 15 min in a 10KC Raytheon sonic oscillator. The 105,000 X g supernatant derived from this sonicate was subjected to ammonium sulphate treatment at 0° and the material precipitating between 40 and 80 per cent saturation was collected by centrifugation, dissolved in 0.001 M mercaptoethanol-0.01 M Tris, pH 7.5, and dialyzed for 4 hr in the cold against 2 changes of the same buffer. This preparation was stored at -20° and used as the enzyme source for the experiments described in this report; it appeared to be stable for several weeks.

Assays for labeled RNA. The presence of ^{35}S-labeled RNA in the reaction mixture was detected in the following manner. The reaction was stopped by adding 3 ml of unlabeled cysteine (10 μmoles), 1 mg of carrier RNA, and 5 mg of cetyltrimethylammonium bromide (CTAB); the precipitate that formed after standing for 1 hr at 0° was collected by centrifugation, resuspended in 0.5 ml of 2 M NaCl-0.005 M Tris, pH 8, and 2 vol of ethanol were added. After 1 hr at -20°, the precipitate was collected by centrifugation, resuspended in 0.5 ml of 0.005 M NaCl-0.5 M Tris, pH 10, placed in a 37° bath for 45 min, and then phenol-extracted once. The RNA was recovered from the aqueous phase by precipitation with 2 vol of cold ethanol and plated for radioactivity determination by Q gas counting after dissolving in 0.01 N NH_4OH. In those mixtures where no enzyme was present during incubation, E. coli extract was added immediately before the CTAB.

RESULTS

Lipsett[1] demonstrated that the presence of thiouridylate in E. coli sRNA is responsible for an additional absorption peak in the spectrum of this ribonucleic acid, exhibiting a maximum at 335 mμ, and an absorption approximately 1.5 per cent of that at 260 mμ. Lipsett[2] also reported that 4-thiouridine is desulfated and converted to uracil (plus other products) when treated with periodate. It was observed that when E. coli sRNA itself was subjected to periodate treatment the 335 mμ absorption peak disappeared, suggesting that a similar desulfuration had taken place (Fig. 1). If thiolation of pyrimidines occurs after nucleic acid polymerization, the detection of such an activity in cell extracts might be facilitated by using periodate-treated sRNA as the sulfur acceptor.

Table 1 shows that a significant amount of radioactivity becomes associated with the RNA fraction when ^{35}S-labeled cysteine is incubated in a complete reaction mixture. Under the conditions employed, ^{35}S incorporation into RNA requires the addition of enzyme; E. coli extracts heated at 100° are inactive. Optimal labeling of the RNA fraction requires added ATP, magnesium, and acceptor sRNA; the thiolation reaction, however, is only partially dependent upon these additions, suggesting that there may be limiting quantities of these components in the crude enzyme. An absolute requirement for divalent metal ion is suggested by the fact that in the absence of Mg^{++}, the incorporation of ^{35}S can be lowered to base line levels by the addition of EDTA. More recent experiments with E. coli extracts that have been treated with streptomycin sulfate show a greater dependency on added RNA for thio-transferase activity, presumably due to removal of endogenous sRNA. The data in Table 1 also suggest that the label incorporated

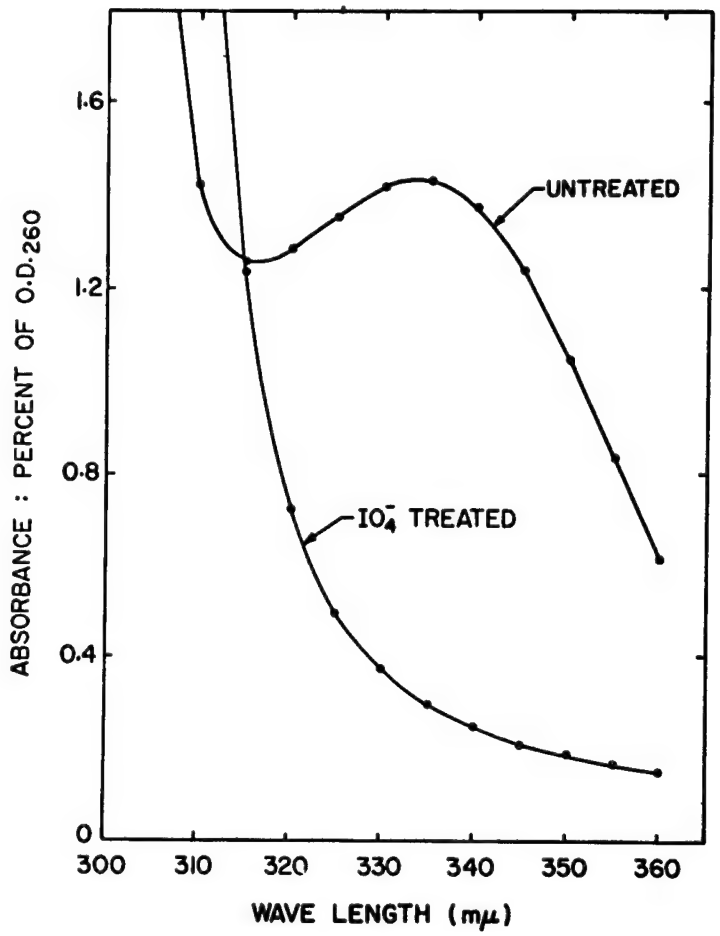

Figure 1. Effect of $NaIO_4$ treatment on the ultraviolet spectra of E. coli K12 sRNA. The periodate treatment was carried out as described under Methods. For the wavelength range shown, the absorbancies for the treated and untreated RNA preparations are expressed as % of the corresponding absorbance at 260 mμ which is not detectably altered by periodate.

is not cysteine itself, since incubation with ^{14}C-cysteine instead of ^{35}S-cysteine produces only base line levels of radioactivity in the RNA fraction. Actinomycin D, DNase, puromycin and chloramphenicol have no detectable effect on the system, but the addition of ribonuclease (pancreatic and T_1) greatly reduces label incorporation.

When hydrolyzed with alkali or exposed to pancreatic ribonuclease, the ^{35}S-labeled reaction product was no longer precipitable with 2 vol of cold ethanol, but was largely adsorbable onto charcoal, and could be removed by extraction with 50 per cent ammoniacal ethanol. Figures 2A and 2B compare the chromatographic behavior of unreacted labeled substrate, and an alkaline digest of the isolated labeled reaction product on DEAE-cellulose. When a mixture of ^{35}S-cysteine, the four major 2'(3')NMPs, and 4-thioUMP was applied to a column of DEAE-cellulose in 0.02 M NH_4HCO_3, pH 8.6, only half of the radioactive amino acid, but all of the ultraviolet absorbing material, was adsorbed. Under the chromatographic conditions employed, the adsorbed ^{35}S-material was eluted in two primary peaks in the same region as the major nucleotides (A_{260}), and no radioactivity was found associated with the well separated band of 4-thioUMP

Table 1

REQUIREMENTS FOR TRANSFER OF LABEL FROM CYSTEINE TO RNA

Expt. No.	Reaction system	Type of label	Counts in RNA fraction (cpm)
1	Zero time	^{35}S-cysteine	376
	Complete	^{35}S-cysteine	2,389
	Complete (heated enzyme)	^{35}S-cysteine	364
	Omit enzyme	^{35}S-cysteine	364
	Omit IO_4^--sRNA	^{35}S-cysteine	1,225
	Omit ATP	^{35}S-cysteine	1,370
	Omit Mg^{++}	^{35}S-cysteine	780
	Omit Mg^{++}, plus EDTA	^{35}S-cysteine	357
2	Zero time	^{35}S-cysteine	377
	Complete	^{35}S-cysteine	1,622
	Zero time	^{14}C-cysteine	216
	Complete	^{14}C-cysteine	293

For experiment 1, the complete system (0.50 ml) contained 50 μmoles of Tris buffer (pH 8.5), 0.8 μmoles of ATP, 5 μmoles of 2-mercaptoethanol, 1.5 μmoles of $MgCl_2$, 250 μg of IO_4^--sRNA (E. coli K12), 0.05 μmoles of cysteine-^{35}S (11.9 x 10^6 cpm per μmole), and 2.5 mg of enzyme. Where indicated, 2.5 μmoles of EDTA were added. Incubation was for 10 min at 37°. In experiment 2, the conditions used were the same as above except that the cysteine-^{35}S had a specific activity of 9.7 x 10^6 cpm per μmole and was replaced where indicated by an equal amount of cysteine-^{14}C, adjusted to the same specific activity.

(Figure 2A, A_{320}). Upon similar treatment of the hydrolyzed reaction product, 86 per cent of the total radioactivity applied to the column was adsorbed onto DEAE-cellulose; half of the adsorbed radioactivity was eluted in the region of 260 mμ absorbing material, and the remainder appeared in fractions coincident with and immediately adjacent to 4-thioUMP (Figure 2B). At the present time, we do not know whether the two overlapping radioactive peaks in the thioUMP region represent different thiolated nucleotides. It is also possible that some of the radioactive material that elutes in the region of the 2'(3')-nucleoside monophosphates may include products other than cysteine or cystine.

Figure 3 shows the effect of varying the enzyme concentration on incorporation of label into the RNA fraction. Under the conditions employed, ^{35}S incorporation increases linearly with addition of extract up to 0.15 ml; further enzyme addition results in higher levels of incorporation, which deviate from linearity and show no apparent end point or saturation (curve A). Since the crude extract itself contains some acceptor (Table 1), it might be expected that increased enzyme additions would give results similar to those obtained. If the values in curve A are adjusted to correct for the contribution of the endogenous acceptor (by determining the amount of ^{35}S incorporated in the absence of added IO_4^--sRNA) then the incorporation data show linearity with respect to enzyme concentration up to 0.15 ml of extract, at which point saturation is obtained (curve B).

The kinetics of the reaction at two different pH values are shown in Figure 4. In the presence of enzyme and at pH 8.5 (which appears to be optimal), ^{35}S incorporation exhibits roughly

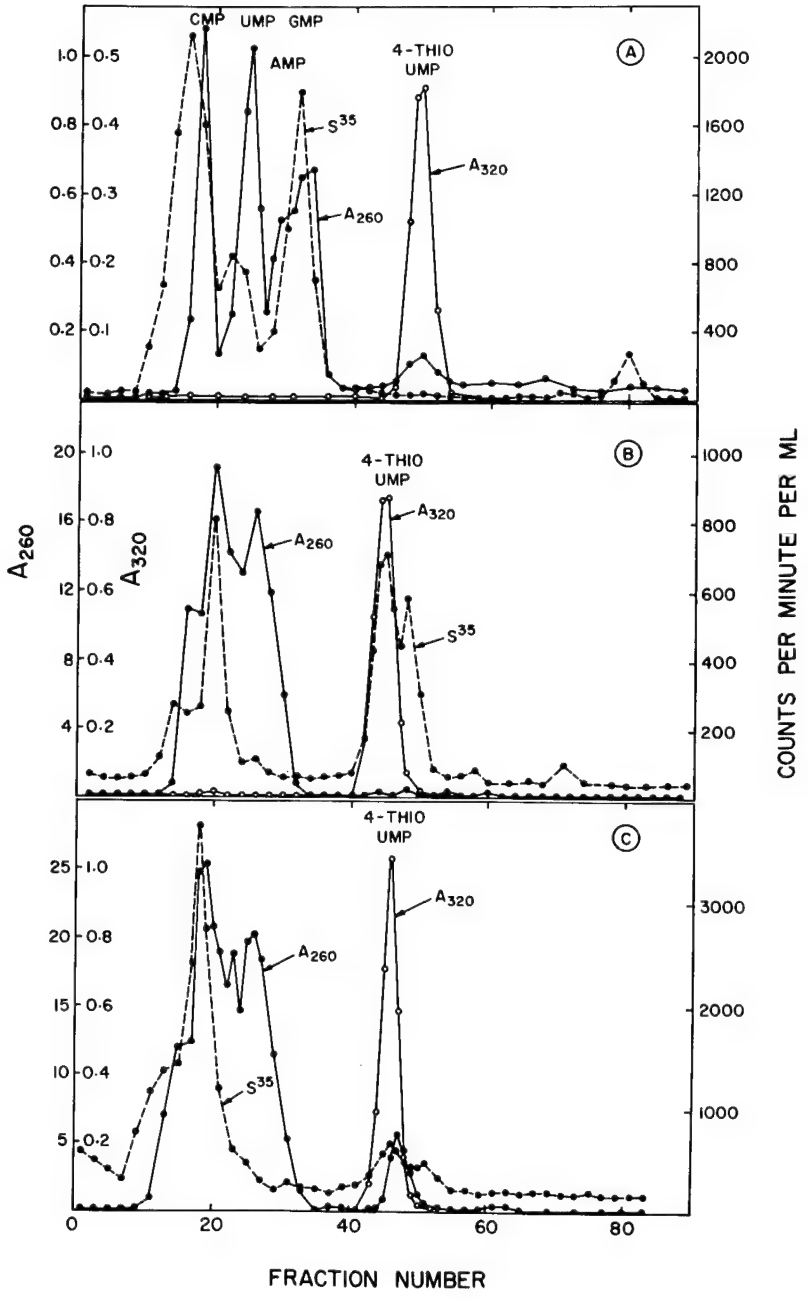

Figure 2. Chromatography of cysteine-^{35}S and alkaline digests of ^{35}S-labeled reaction product. The chromatography conditions employed were similar to those described by Lipsett.[2] The material was applied to a column of DEAE-cellulose (4 g dry weight) in 0.01 M NH_4HCO_3 (pH 8.6), washed with 100 ml of 0.02 M of the same buffer and eluted with 400 ml of a linear gradient of NH_4HCO_3 (pH 8.6) from 0.05 M to 0.25 M with 7 M urea present. One ml from each fraction (4.5 ml) was added to 16 ml of Bray's scintillant and counted in a Nuclear Chicago Scintillometer. The material chromatographed in each of the above runs were as follows. (A) A mixture of cysteine-^{35}S, 4-thioUMP and the 2'(3')-derivatives of CMP, UMP, AMP, and GMP; (B) an alkaline hydrolysate of the pH 8.5 enzymatic reaction product (cysteine-^{35}S as substrate and E. coli K12 IO_4^--sRNA as acceptor) plus 4-thioUMP added as carrier; and (C) the same as in B except the reaction was carried out at pH 6.6 without enzyme. The total recovery of counts and carrier nucleotide was greater than 90 per cent in each case.

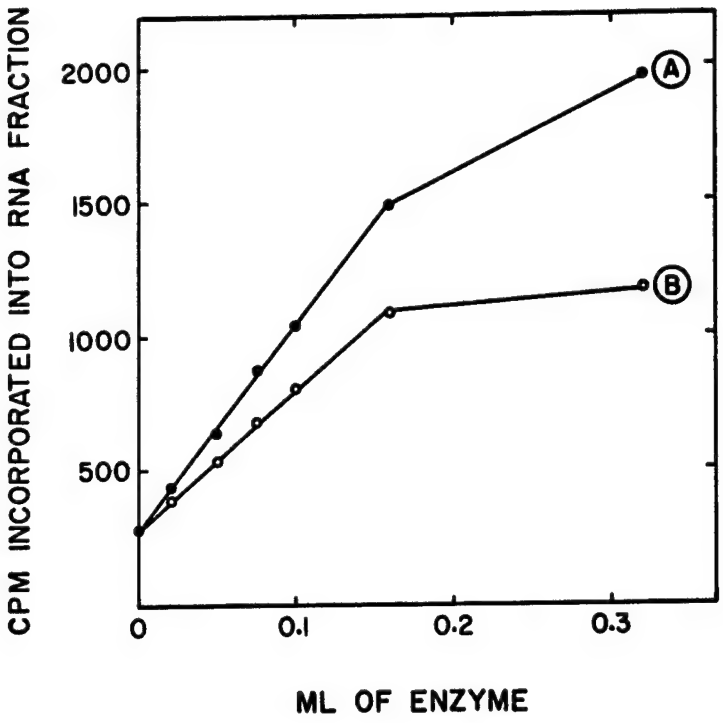

Figure 3. Enzyme concentration and labeled sRNA formation. The reaction mixture (0.50 ml) and the conditions employed were the same as shown for Table 1 except that 133 μg of IO_4^--sRNA, and various amounts of E. coli enzyme (11 mg per ml) were used. Curve A represents the observed counts incorporated and curve B represents the same data corrected for the counts incorporated in the absence of added IO_4^--sRNA which was approximately 256 cpm (over base line) for 0.10 ml of enzyme. The data have not been corrected for zero time wash-out controls.

linear kinetics for the first 15-20 min of reaction, and thereafter increases at a slower rate (Figure 4A). In the absence of enzyme and at the same pH, a significant amount of label is incorporated into the RNA fraction upon incubation for extended periods. The initial rate of the nonenzymatic incorporation at pH 8.5 is far less than that observed when enzyme is present, but after 20 min the enzymatic and nonenzymatic rates appear to be fairly similar. When phosphate buffer, pH 6.6, was used in place of the higher pH Tris, an active incorporation of ^{35}S was found in the absence of enzyme; vessels that contained enzyme showed no higher levels of incorporation, indicating that at this lowered pH the incorporation was entirely of a nonenzymatic nature (Figure 4B). By using Tris buffers of pH values lower than 8.5, one also finds an increase in the nonenzymatic, and decrease in the enzymatic reaction. In the absence of ATP and Mg^{++}, with or without EDTA, the nonenzymatic reaction is only slightly reduced. Chromatographic analysis of an alkaline digest of the labeled nonenzymatic reaction product indicated that not more than 10 per cent of the total radioactivity recovered was associated with 4-thio-UMP; most of the label was found in the early fractions (Figure 2C). In contrast to the enzymatic reaction at pH 8.5, the nonenzymatic pH 6.6 reaction leads to significant incorporation of ^{14}C-cysteine into the RNA fraction relative to the zero time.

Although the enzyme preparation contains endogenous acceptor, it is present in limited quan-

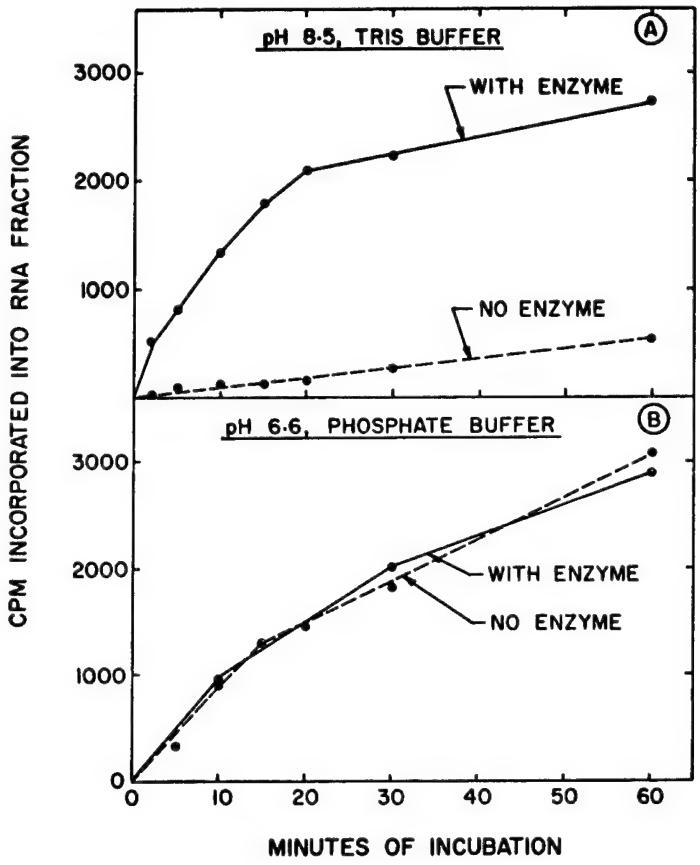

Figure 4. Kinetics of the enzymatic and nonenzymatic incorporation of ^{35}S into the RNA fraction at two different pH's. The reaction mixture was essentially the same as described for Table 1 except that the mixtures in B contained 50 μmoles of potassium phosphate (pH 6.6) and where indicated 1.65 mg of enzyme. Incubation was carried out at 37° for the time periods shown above. The data shown have been corrected for zero time wash-out controls (200 cpm at pH 8.5 and 600 cpm at pH 6.6).

tities, and added acceptor is required for optimal incorporation. Figure 5 shows that for a given amount of E. coli extract there is an increase in ^{35}S incorporation with the amount of IO_4^--sRNA added. Although the data presented here show an apparent saturation level at approximately 0.25 mg of RNA, a further increase in label incorporation occurs when larger quantities of IO_4^--sRNA are used (1 mg or more) with the same amount of enzyme. The exact reason for this is not clear; we have observed, however, that the rate of the nonenzymatic reaction (at pH 8.5) is dependent upon the amount of RNA acceptor present. The nonenzymatic activity at this pH is negligible at low RNA concentrations, but becomes significant at higher levels.

One question of immediate interest is the type of specificity exhibited by the E. coli Q13 enzyme with respect to the ability of various nucleic acids to act as ^{35}S-acceptors. So far, the only nucleic acids that show detectable acceptor activity, under the assay conditions employed here, are those derived from the soluble supernatant of cell extracts (Table 2). E. coli ribosomal RNA and DNA (native or denatured) and the synthetic homopolymers containing either uridine, adenine, or cytidine (IO_4^--treated) show no higher levels of ^{35}S incorporation into the

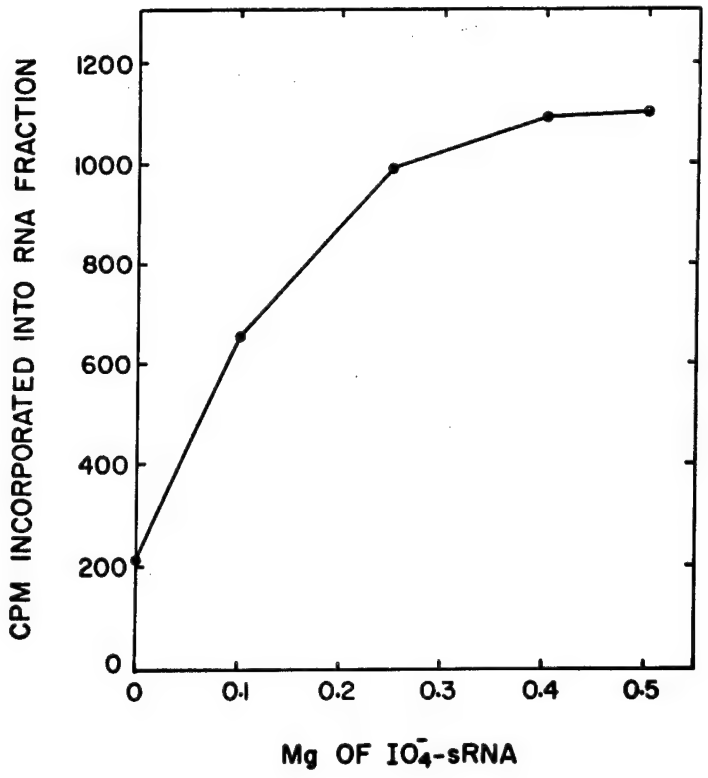

Figure 5. sRNA dependence of RNA thiolase activity. The reaction mixture and conditions employed were essentially the same as shown in Table 1 except that the amount of IO_4^--sRNA was varied and 1.1 mg of enzyme was used. The data have been corrected for zero time wash-out levels (200 cpm).

RNA fraction, and the level is usually lower, than in control vessels from which RNA was omitted. Nonperiodate-treated poly C has consistently shown a slight but significant stimulation of ^{35}S incorporation over control values; we have no further information on this reaction. All of the different soluble RNAs tested (treated or untreated with IO_4^-) show significant activity as sulfur acceptors. Periodate treatment of the various soluble RNAs tested enhances their acceptor ability, except in the case of yeast sRNA, where a significant reduction seems to occur.

DISCUSSION

The central role of transfer RNAs in protein synthesis lends particular importance to an understanding of their formation. It seems reasonable to assume that the "minor" nucleotides in transfer RNA are involved in conferring the type of biochemical specificity required for at least some of the special functions of this ribopolynucleotide. For each transfer RNA, specific sites within the polymerized molecule must be available for (a) recognition of the appropriate activating enzyme for amino acyl esterification, (b) attachment to ribosomes, (c) recognition of the proper messenger codon, and (d) possibly other functions involved with control mechanisms. The exact role of the "minor" nucleotides in these various functions is, as yet, unclear.

In the work presented here, we have shown that E. coli extracts will catalyze the transfer of sulfur from cysteine to ribonucleic acid, and that this transfer requires ATP, a divalent metal

Table 2

THE ABILITY OF DIFFERENT NUCLEIC ACIDS TO SERVE AS ^{35}S-ACCEPTORS

Reaction mixture	Type of RNA used	^{35}S incorporated into RNA fraction	
		Untreated nucleic acid (cpm)	IO_4^- treated nucleic acid (cpm)
Omit enzyme	E. coli K12 sRNA	303	400
Omit RNA		885	-
Complete	E. coli K12 sRNA	1,348	2,045
Complete	E. coli B sRNA	1,078	1,842
Complete	Yeast sRNA	2,516	1,865
Complete	Rat liver sRNA	1,498	1,722
Complete	E. coli Rb RNA	617	794
Complete	E. coli DNA (native)	911	763
Complete	E. coli DNA (heat-denatured)	734	665
Complete	Poly U	579	534
Complete	Poly A	647	576
Complete	Poly C	1,279	542

The complete system (0.50 ml) and conditions of reaction was the same as indicated for Table 1 except that various nucleic acid preparations (250 μg each) were used where indicated, the specific activity of the cysteine-^{35}S was 12.9×10^6 cpm per μmole, and 2.1 mg of enzyme was added.

ion, and RNA derived from soluble cell extracts. Although sRNA as isolated from the cell serves as an acceptor for sulfur, the prior treatment of the RNA with $NaIO_4$ enhances its ability to act as an acceptor (except for yeast sRNA). The ^{35}S-labeled product, derived from ^{35}S-cysteine, is susceptible to degradation with alkali and pancreatic RNase, and the bulk of the labeled hydrolyzed material has chemical properties similar to those of nucleotides. When the labeled product is subjected to alkaline hydrolysis and then chromatographed on DEAE-cellulose, the distribution of radioactivity upon elution with a salt gradient in 7 M urea suggests that several products have been formed; one of these appears to be 4-thiouridylate, although further chemical identification is required for certainty. Cysteine carbon is not significantly incorporated in the enzymatic reaction studied.

At pH 8.5, the transfer of ^{35}S from cysteine to RNA is primarily enzymatic for incubation periods up to 20 min, and at relatively low acceptor RNA concentrations. For longer periods of incubation, or at high RNA concentration, one observes significant ^{35}S incorporation in the absence of enzyme. At higher hydrogen ion concentrations, the nonenzymatic transfer of label to the RNA fraction becomes more active; at pH 6.6, the transfer is entirely of a nonenzymatic nature. The nonenzymatic reaction is largely unaffected by the addition of ATP and metal ion, and has only been observed with E. coli K12 IO_4^--sRNA. Analysis of the nonenzymatic reaction product indicates the formation of some 4-thioUMP, but this forms a much smaller percentage of the total product than in the case of the enzymatic reaction. In the absence of enzyme, much of the ^{35}S associated with the RNA fraction appears to be accompanied by an equivalent amount of cysteine carbon; whether this represents a chemical association of intact cysteine with RNA, or

some other artifact, occurring less markedly at high pH, is unknown. It seems unlikely that the cysteinyl-sRNA, active in protein synthesis, could be present in the product.

The lack of demonstrable enzymatic transfer of sulfur from cysteine to nucleic acids other than sRNA suggests that a high degree of specificity with respect to base sequence and/or secondary structure of the acceptor is required for thiolase activity. This type of specificity also suggests, but does not prove, that the sulfur containing nucleotides in sRNA arise by thiolation after polynucleotide formation. The lack of inhibition of the thiolase reaction by DNase and actinomycin D, which are inhibitors of RNA polymerase activity, is consistent with this hypothesis. Precedents for chemical modification of nucleotides at the polynucleotide level have already been cited. It is interesting to note that the sRNAs from both yeast and rat liver served as ^{35}S acceptors with the E. coli enzyme, in vitro; yet these particular RNAs show no absorption maximum at 335 mμ, and presumably contain no 4-thiouridylate residues. Although we have not analyzed the reaction products formed when these two RNAs serve as sulfur acceptors, it is quite possible that appropriate sites are available for in vitro uridine-thiolation but are not thiolated in vivo because the necessary enzyme is absent from these species.

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THE INFLUENCE OF POLYCYCLIC AROMATIC HYDROCARBONS ON BACTERIOPHAGE DEVELOPMENT. II.

By

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A recent publication from this laboratory[1] described the inhibitory effect of certain aromatics on the replication of single-stranded RNA† and DNA bacterial viruses using the isolated nucleic acid as the infectious agent. At the same time, it was observed that most of the aromatics that exhibited this effect were also carcinogenic. The viral replication response to hydrocarbons requires the use of infected bacterial spheroplasts; no effect is observed with intact infected bacteria. Although the mechanism by which the aromatics exert their inhibitory influence is unclear, it appears that the active hydrocarbons do not inhibit the infectious process itself or the release of newly formed viruses, nor do they induce new enzymes inhibitory for viral production.

The present communication provides further information on the nature of the hydrocarbon response in infected and noninfected Escherichia coli spheroplasts. A method is described for preparing infected spheroplasts from cells preinfected with phage which permits one to assay the hydrocarbon effect on the replication of both single- and double-stranded viruses whereby significant differences are observed. The present study demonstrates the inhibition of viral nucleic acid and protein synthesis by 7,12-DNBA, and, in contrast to one of our earlier observations, we now report that 7,12-DMBA inhibits nucleic acid and protein synthesis in the bacterial host itself. The protective effect of various aromatics on 7,12-DMBA-induced viral inhibition is also reported.

METHODS

Bacterial viruses and infectious RNA. MS2 and lambda (λ) phage were grown on the male strain of E. coli K12W1485; ϕX174 and T4 phage were grown on E. coli C and E. coli B respectively. Infectious MS2 RNA was isolated from MS2 phage by phenol extraction[2] and stored in 5 mM EDTA, pH 7.4, at -20°.

Preparation of infected spheroplasts with chloramphenicol (CA method). The growth of E. coli cells was previously described.[1] To 35 ml of culture at 5×10^5 cells/ml in 3XC medium,[3] 0.7 ml of chloramphenicol (1 mg/ml) and 0.05 ml of 1M $CaCl_2$ were added, and the mixture was allowed to stand for 5 min. Bacteriophage was then added at a ratio of 10 PFU per bacterium for MS2, λ, and ϕX174, and 4 PFU per bacterium for T4, and adsorption was allowed to proceed for another 5 min. The mixture was then chilled in ice and centrifuged at 4°. The packed cells were washed once with 10 ml of 3XD medium containing chloramphenicol (20 μg/ml), and

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†The following abbreviations are used: RNA and DNA for ribo- and deoxyribonucleic acid; DNase, deoxyribonuclease; EDTA, disodium ethylenediaminetetraacetate; PFU, plaque-forming unit; HC, hydrocarbon; BA, benz(a)anthracene; MBA, methylbenz(a)anthracene; DMBA, dimethylbenz(a)anthracene.

then suspended in a mixture containing 0.1 ml of chloramphenicol (10 μg), 0.7 ml of 50 per cent sucrose, 0.02 ml of lysozyme (40 μg) and 0.4 ml of 5 mM EDTA. This mixture was incubated at 30° for 6 min, after which 20 ml of cold PAM medium,[4] modified to contain no peptone, was added. The cells were sedimented in the cold by centrifugation and then resuspended in 20 ml of the modified cold PAM medium without chloramphenicol. These infected spheroplasts were used directly for testing the hydrocarbon response (see below).

Preparation of noninfected spheroplasts. E. coli K12W1485 were converted to spheroplasts with lysozyme and EDTA as previously described[1] except that 0.20 ml of 0.25 M Tris, pH 8.1, was substituted for bovine serum albumin during the lysozyme-EDTA treatment and the spheroplasts were used almost immediately after preparation, instead of standing for 1 hr at room temperature.

Hydrocarbon additions to spheroplasts and viral assay. Stock solutions of hydrocarbon were prepared in dimethylformamide. Appropriate dilutions of the aromatic solutions were made in PAM medium for addition to spheroplasts; the dimethylformamide concentration did not exceed 0.4 per cent in the final assay system. Control assays (no hydrocarbon) contained the same amount of solvent. For the "CA method" of infection, the hydrocarbon suspension was added to an appropriate quantity of infected spheroplasts and then incubated at 30°. After certain times of incubation, cells were removed and lysed by shaking with 3XD medium containing $CHCl_3$ and 0.01 M NaCN. They were then assayed for phage titer by the agar layer technique.[5] The indicator bacteria used for the different phages were the strains of E. coli on which the viruses had been grown. For all assays, the tubes containing the infectious mixtures were wrapped in aluminum foil to exclude light.

Studies with radioactive substrates. For studies on ^{14}C-uracil and ^{14}C-amino acid incorporation into nucleic acid and protein, the labeled compounds were added to spheroplasts, and the cells were then treated at appropriate times with 5 per cent trichloroacetic acid. For RNA determinations, the cold-precipitable material was collected by centrifugation, washed several times with cold acid and then collected on millipore filters, dried, and counted. For protein determinations, the acid-precipitable material was treated in a similar manner except that it was also subjected to heating at 90° for 20 min in 5 per cent acid before isotope determination.

Viral RNA polymerase. Cells of the lysis-inhibited male strain of E. coli C3000 were infected with MS2 phage and converted to spheroplasts by the "CA method" described above. The cells were diluted twofold with PAM medium, and, after gentle shaking for 50 min at 30° in the presence or absence of hydrocarbon, collected by centrifugation, rinsed once with 10 per cent sucrose, lysed in a hypotonic medium containing 0.1 M Tris (pH 8.1), 0.01 M mercaptoethanol and 0.001 M EDTA and centrifuged at 30,000 x g. The supernatant collected after centrifugation was assayed for its ability to catalyze the RNA-dependent synthesis of RNA as described by Lodish, Cooper and Zinder.[6]

Radioactive uracil-2-^{14}C was purchased from Schwarz Bioresearch Inc.; ^{14}C-amino acids were obtained from New England Nuclear Corp.; actinomycin D was a gift from Merck and Co., Inc.

RESULTS

The effect of aromatics on single-stranded RNA and DNA phage replication. Table 1 shows the effect of various polycyclic aromatic hydrocarbons on the replication of MS2 and ϕX174 when

Table 1

EFFECT OF VARIOUS HYDROCARBONS ON MS2 AND ϕX174 PHAGE PRODUCTION

Hydrocarbon	MS2 phage		ϕX174 phage		Biological* activity in rats
	Phage titer (PFU/ml x 10^{-7})	Per cent inhibition	Phage titer (PFU/ml x 10^{-7})	Per cent inhibition	
None	410	0	89	0	-
BA	380	7	84	6	-
7,12-DMBA	39	91	22	75	+
6,8-DMBA	38	91	17	81	+
1,12-DMBA	12	97	18	80	-
3,9-DMBA	410	0	88	1	-
4-MBA	390	5	89	0	-
5-MBA	330	19	73	18	-
6-MBA	24	94	12	86	+
Chrysene	410	0	78	12	-

Infectious spheroplasts were prepared by the "CA method." The hydrocarbon assay mixture contained 0.1 ml of the infected spheroplast preparation (see Methods) and 0.9 ml of PAM medium containing 20 μg of the appropriate hydrocarbon as indicated above. After incubation at 30° for 3 and 2 hr for the MS2 and ϕX174 infectious mixtures respectively, the cells were lysed and titrated for whole phage as described under Methods.

*+ produces sarcoma when 2.5 mg of hydrocarbon in 0.5 ml of sesame oil is injected in muscle of rat.

infected spheroplasts are prepared by the "CA method." It may be seen that certain aromatics strongly inhibit virus production while others have little or no effect. Except for the aromatic 1,12-DMBA, which is non-carcinogenic in rat, those hydrocarbons that strongly suppress viral replication also exhibit biological activity in higher organisms. The reverse correlation is equally impressive; hydrocarbons that are inactive as viral inhibitors are also inactive as carcinogenic inducers. These results are similar to those reported previously, when the isolated viral nucleic acids instead of the intact bacteriophage were used as the infecting agents.[1]

Single burst experiments were performed with MS2 infected cells to determine what proportion of the cells still produce virus in the presence of 7,12-DMBA, and whether the efficiency of virus production is altered. These experiments indicate that in the presence of 7,12-DMBA less than half of the infected cells give rise to phage and the viral burst size per infected bacteria which do produce virus is also lower by a factor of more than two. The overall hydrocarbon inhibition on plaque yield, therefore, results from a combination of these two effects.

Protection against the 7,12-DMBA response. When E. coli spheroplasts are pretreated with certain aromatics prior to exposure to 7,12-DMBA, the viral inhibition response is partially masked (Table 2). The aromatics that partially block the 7,12-DMBA inhibition appear to be those that are not inhibitors themselves, and they are not carcinogenic in rat. Remarkably, in one instance when benz(a)anthracene was added after 7,12-DMBA preincubation, protection against viral inhibition was also observed. These results suggest some type of reversible competition between the aromatics for the active sites responsible for bacteriophage replication. The protection phenomenon by various aromatics on 7,12-DMBA-induced viral inhibition is ex-

Table 2

PROTECTION AGAINST 7,12-DMBA INHIBITION OF MS2 REPLICATION BY VARIOUS AROMATICS

Aromatic treatment of spheroplasts prior to infection with MS2 RNA		
First 30 min	Second 60 min	PFU/ml
None	None	7,690
None	7,12-DMBA (25 μg/ml)	149
7,12-DMBA (2.5 μg/ml)	7,12-DMBA (25 μg/ml)	111
BA (25.0 μg/ml)	7,12-DMBA (25 μg/ml)	3,590
3,9-DMBA (25.0 μg/ml)	7,12-DMBA (25 μg/ml)	4,785
6,8-DMBA (25.0 μg/ml)	7,12-DMBA (25 μg/ml)	280
Chrysene (25.0 μg/ml)	7,12-DMBA (25 μg/ml)	2,875
6-Aminochrysene (25.0 μg/ml)	7,12-DMBA (25 μg/ml)	257
7,12-DMBA (25.0 μg/ml)	BA (25 μg/ml)	3,000

E. coli spheroplasts were incubated with various hydrocarbons (concentrations shown above) at 30° prior to infection. The hydrocarbons incubated with the cells for the first 30 min are shown in the first column. 7,12-DMBA or BA was then added to the mixtures and incubation continued for another 60 min. MS2 RNA (50 mμg in 0.50 ml) was then added to 0.50 ml of the hydrocarbon-treated spheroplasts and the infectious mixtures assayed for PFU after 20 min incubation at 30°. Each figure represents the average of duplicate assays.

tremely interesting, since a similar effect has been reported for DMBA-induced cancers in rats.[7,8]

Viral nucleic acid synthesis. MS2 RNA synthesis was observed by determining the rate of ^{14}C-uracil incorporation into acid-insoluble material in the presence of actinomycin D (Figure 1). The antibiotic was included in the infected spheroplast mixture to suppress the DNA-directed RNA synthesis of the host; its effectiveness is demonstrated by the low level of uracil incorporation obtained with noninfected cells. On the other hand, MS2 infected cells show an active incorporation of labeled uracil into the RNA fraction in the presence of actinomycin D, suggesting that the rate of this incorporation reflects the rate of viral RNA synthesis. Under the same conditions, 7,12-DMBA lowers the rate of uracil incorporation. Assays for the appearance of infectious RNA throughout the period of viral development also indicate that the rate and extent of viral RNA synthesis are similarly suppressed by 7,12-DMBA. Because of the reduced RNA synthesis observed, assays for viral RNA polymerase activity (induced by virus infection) were performed on extracts of infected cells. Figure 2 shows the kinetics of UT^{32}P incorporation into RNA catalyzed by a 30,000 x g extract prepared from MS2 infected spheroplasts in the presence of actinomycin D and pancreatic DNase. The low level of UT^{32}P incorporation observed with extracts from noninfected cells indicates the effectiveness of the antibiotic and DNase in blocking E. coli RNA polymerase activity (lower curve). Extracts prepared from MS2 infected cells, exposed to benz(a)anthracene or 7,12-DMBA, catalyzed less label incorporation than extracts from cells that had not been exposed to the aromatics; 7,12-DMBA is particularly effective in this respect.

The effect of hydrocarbons on viral protein synthesis. Figure 3 illustrates the effect of

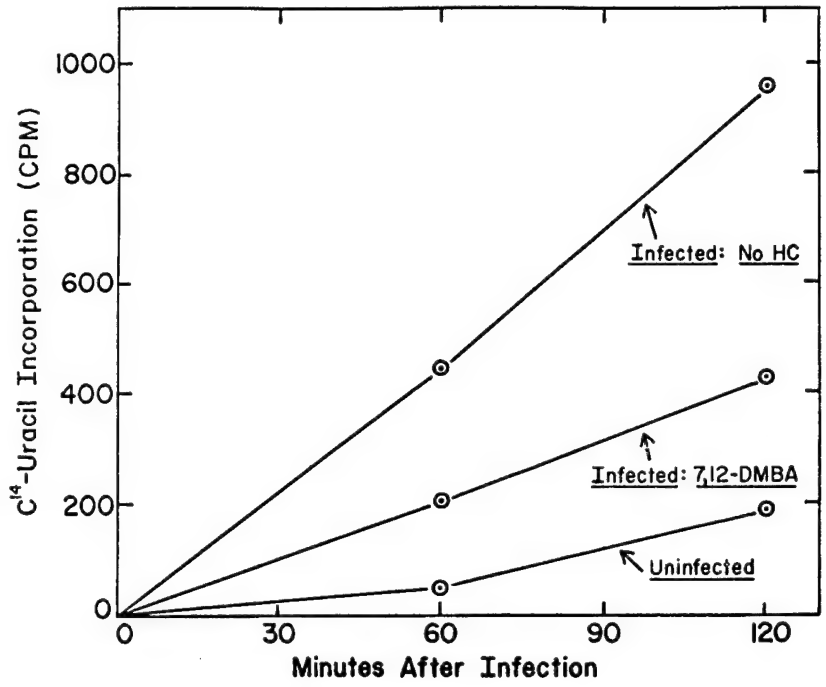

Figure 1. Effect of 7,12-DMBA on ^{14}C-uracil incorporation by MS2 infected cells. The reaction mixture (4.84 ml) in the modified PAM medium contained E. coli spheroplasts (approximately 4 x 10^8 cells, either infected or noninfected); 97 μg of 7,12-DMBA; 0.1 μmole of uracil-2-^{14}C (45 mc per μmole); and 100 μg of actinomycin D. The mixtures were incubated at 30° and 0.60 ml samples were withdrawn at various times and the ^{14}C content in the acid-precipitable fraction determined as described under Methods. Phage titers on separate samples showed a 7,12-DMBA inhibition of 61 per cent at 240 min after infection.

7,12-DMBA on the incorporation of a mixture of labeled amino acids into the protein fraction of MS2 infected spheroplasts. It is apparent that the rate and extent of labeled amino acids incorporated is significantly lowered by the hydrocarbon. It is probable that some of the labeled protein represents host protein synthesis, even though actinomycin D was added to the spheroplast mixture. Studies with MS2 antisera suggest a similar hydrocarbon response.

Serum blocking experiments, as described by De Mars,[9] offer an extremely sensitive method for detecting viral protein synthesis. This procedure involves adding a slight excess of phage antiserum to samples of phage antigen (serum blocking material), and, after antiserum-antigen interaction, assaying for unreacted antiserum by its ability to inactivate a known quantity of test phage. The extent of test phage survival reflects the amount of unreacted antiserum and provides an indirect measure of the quantity of antigen (viral protein) in the unknown samples. Table 3 shows that in the absence of hydrocarbon, or in the presence of benz(a)anthracene, a sufficient amount of viral protein is made 60 min after MS2 infection, which almost completely "blocks" the inactivation of test phage by the antiserum. In the presence of 7,12-DMBA, no appreciable phage antigen is synthesized 60 min after infection since the test phage is inactivated to nearly the same extent as the controls (zero time) where no phage antigen is present.*

*Since whole phage antiserum may not interact with phage protein subunits unless they are assembled into a structure comparable to that found in the intact phage, this experiment does not prove that 7,12-DMBA inhibits phage protein formation; however, it is consistent with the amino acid isotope incorporation data.

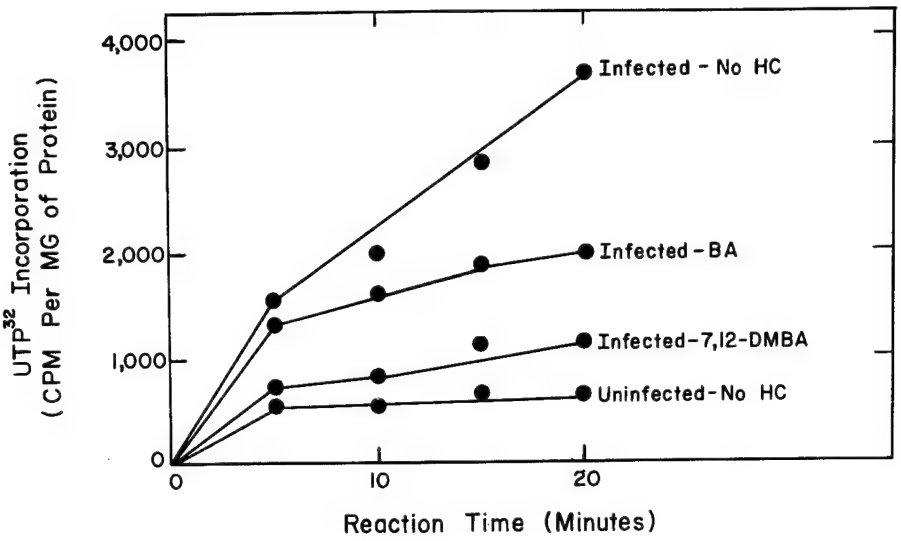

Figure 2. Effect of aromatics on MS2 RNA polymerase activity. The preparation of noninfected and MS2 infected spheroplast extracts, treated or nontreated with hydrocarbons, is described under Methods. The reaction mixture (2.6 ml) contained 300 μmoles of phosphate buffer (pH 7.5); 24 μmoles of $MgCl_2$; 60 μmoles of mercaptoethanol; 3.3 μmoles each of ATP, CTP and GTP; 2.9 μmoles of UTP-α-^{32}P (13 x 10^6 CPM per μmole); 12 μmoles of phosphoenol pyruvate; 24 μg of pyruvate kinase; 12 μg of DNase; 30 μg of actinomycin D and 0.60 ml of a 30,000 x g spheroplast extract. Aliquots were removed at various times and the label incorporated into the acid-precipitable fraction was determined. The incorporation data were adjusted to correct for the slight differences in protein content of each sample assayed.

Similar results were obtained when the cells were lysed 2 hr after infection.

The effect of aromatics on the replication of double-stranded DNA viruses. The "CA method" of preparing infected spheroplasts allows an examination of the hydrocarbon response to double-stranded as well as single-stranded viral replication. If the inhibition of bacteriophage replication by aromatics is a general phenomenon one would expect the replication of all viruses to be similarly affected. Table 4 shows the effect of the hydrocarbons previously examined on the replication of the double-stranded bacteriophages λ and T4. The results indicate that some of the aromatics that were strong inhibitors for the single-stranded viruses now appear to be either less effective (i.e., 7,12-DMBA and 1,12-DMBA) or show little significant activity (i.e., 6,8-DMBA and 6-MBA). The previous correlation between hydrocarbons active as viral inhibitors and those active as carcinogens no longer seems to hold when the double-stranded viruses are employed as the infecting agents.

The influence of aromatics on host nucleic acid and protein synthesis. In a previous communication,[1] we indicated that the aromatics exerted no observable effects on nucleic acid and protein synthesis in E. coli spheroplasts. Since the assembly of viral constituents involves the use of the host's biochemical machinery, it seemed important to confirm these findings. When spheroplasts were used immediately after preparation, instead of after standing for a few hours as in the earlier studies, 7,12-DMBA (but not benz(a)anthracene) significantly lowered ^{14}C-uracil and ^{14}C-leucine incorporation into an acid-precipitable fraction (Figure 4). Many differ-

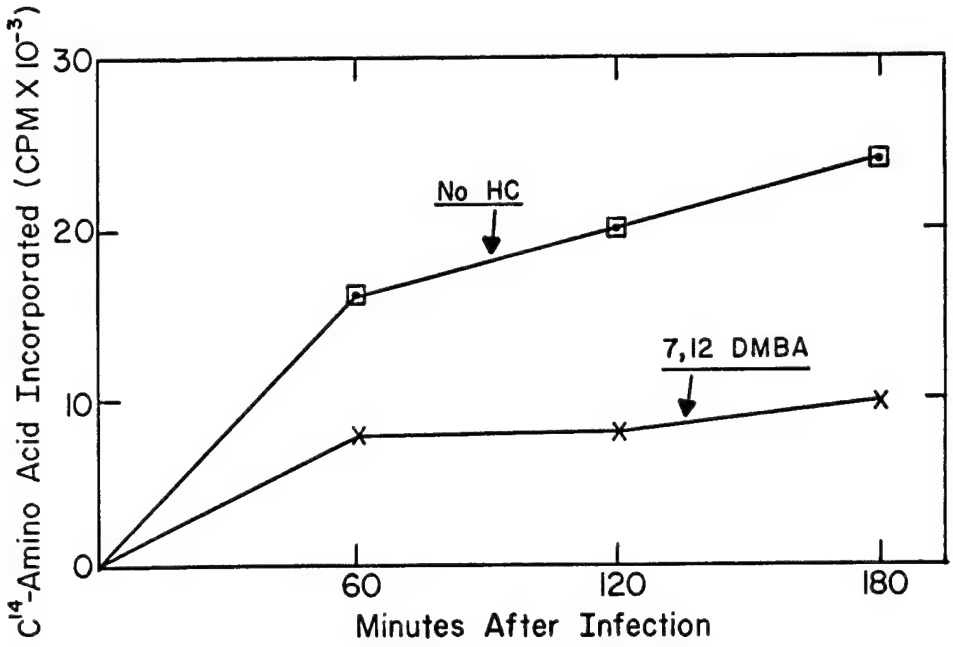

Figure 3. Effect of 7,12-DMBA on the incorporation of ^{14}C-amino acids by MS2 infected cells. The reaction mixture (1 ml) in the modified PAM medium contained E. coli spheroplasts (approximately 1 x 10^8 cells, either infected or noninfected); 5 μc each of ^{14}C-leucine, ^{14}C-valine and ^{14}C-serine; 20 μg of 7,12-DMBA and 10 μg of actinomycin D. The mixtures were incubated at 30° and 0.2 ml samples were withdrawn at various times and the incorporation of label into protein was determined as indicated under Methods. Phage titer determinations on separate samples, 3 hr after infection, showed a 90 per cent inhibition by 7,12-DMBA.

ent experiments with E. coli spheroplasts now confirm this observation and can be summarized as follows: (1) the active aromatics that inhibit bacterial nucleic acid and protein synthesis appear to be the same as those that inhibit virus formation; (2) the presence of chloramphenicol does not diminish the 7,12-DMBA inhibition of uracil incorporation into RNA, suggesting that this effect is independent of bacterial protein synthesis; (3) 7,12-DMBA inhibits the incorporation of ^{32}Pi into acid-soluble organic phosphate (charcoal adsorbable) and into RNA and DNA; and (4) 7,12-DMBA inhibits the incorporation of various amino acids as well as leucine (e.g., lysine, isoleucine and phenylalanine) into protein, although there is little or no change in serine incorporation. On occasion, 7,12-DMBA has shown marked inhibiting effects on the polyuridylate-directed assembly of ^{14}C-phenylalanine polypeptides in cell-free protein synthesizing systems with E. coli extracts, although this effect has not been consistently reproducible.

DISCUSSION

The present study confirms and extends some of our earlier observations but also confronts us with some perplexing problems. The two infectious procedures used for the hydrocarbon assay, (a) spheroplast infection with viral nucleic acids[1] and (b) the conversion of infected bacteria to spheroplasts, show similar inhibitions with the same aromatics for cells infected with single-stranded viruses. Some differences in the sensitivity to certain hydrocarbons have been observed,

Table 3

EFFECT OF HYDROCARBONS ON THE PRODUCTION OF SERUM BLOCKING MATERIAL BY MS2 INFECTED CELLS

Serum blocking material	MS2 antiserum	Test MS2	Test phage survival	
			PFU	Per cent inactivated
None	0	+	739	0
None	+	+	9	99
No hydrocarbon (0' lysate)	+	+	11	99
No hydrocarbon (60' lysate)	+	+	626	15
BA (0' lysate)	+	+	9	99
BA (60' lysate)	+	+	572	23
7,12-DMBA (0' lysate)	+	+	15	98
7,12-DMBA (60' lysate)	+	+	91	88

MS2 antiserum was prepared from rabbits which had been injected with whole MS2 phage for a period of one month. The K value for the stock antiserum (velocity constant for virus inactivation at 37°) was 1.5×10^4 per min. MS2 infected spheroplasts (CA Method), after incubation in the absence or presence of hydrocarbon for 60 min, were lysed by freezing and thawing. The lysed cells were exposed to ultraviolet light for 5 hr to inactivate virus; no significant infectivity was detected after this treatment. The ultraviolet-treated lysate (serum blocking material) was incubated for 12 hr with MS2 antiserum (final concentration 5×10^{-4} dilution of stock), test MS2 phage was added and incubated for another 60 min and then assayed for surviving virus.

Table 4

EFFECT OF VARIOUS HYDROCARBONS ON PHAGE λ AND T4 PRODUCTION

Hydrocarbon	Phage λ		T4		Biological* activity in rats
	Phage titer (PFU/ml x 10^{-5})	Per cent inhibition	Phage titer (PFU x 10^{-7})	Per cent inhibition	
None	88	0	22	0	-
BA	85	3	24	0	-
7,12-DMBA	39	56	12	45	+
6,8-DMBA	90	0	20	9	+
1,12-DMBA	50	43	17	23	-
3,9-DMBA	84	5	15	32	-
4-MBA	77	12	18	18	-
5-MBA	89	0	20	9	-
6-MBA	66	25	19	14	+
Chrysene	79	10	23	0	-

Spheroplasts infected with λ and T4 phage were prepared by the CA Method. The assay mixtures and conditions were the same as indicated in Table 1 except that the incubation time of the infectious-hydrocarbon mixtures was for 2 hr.

*+ produces sarcoma when 2.5 mg of hydrocarbon in 0.5 ml of sesame oil is injected in muscle of rat.

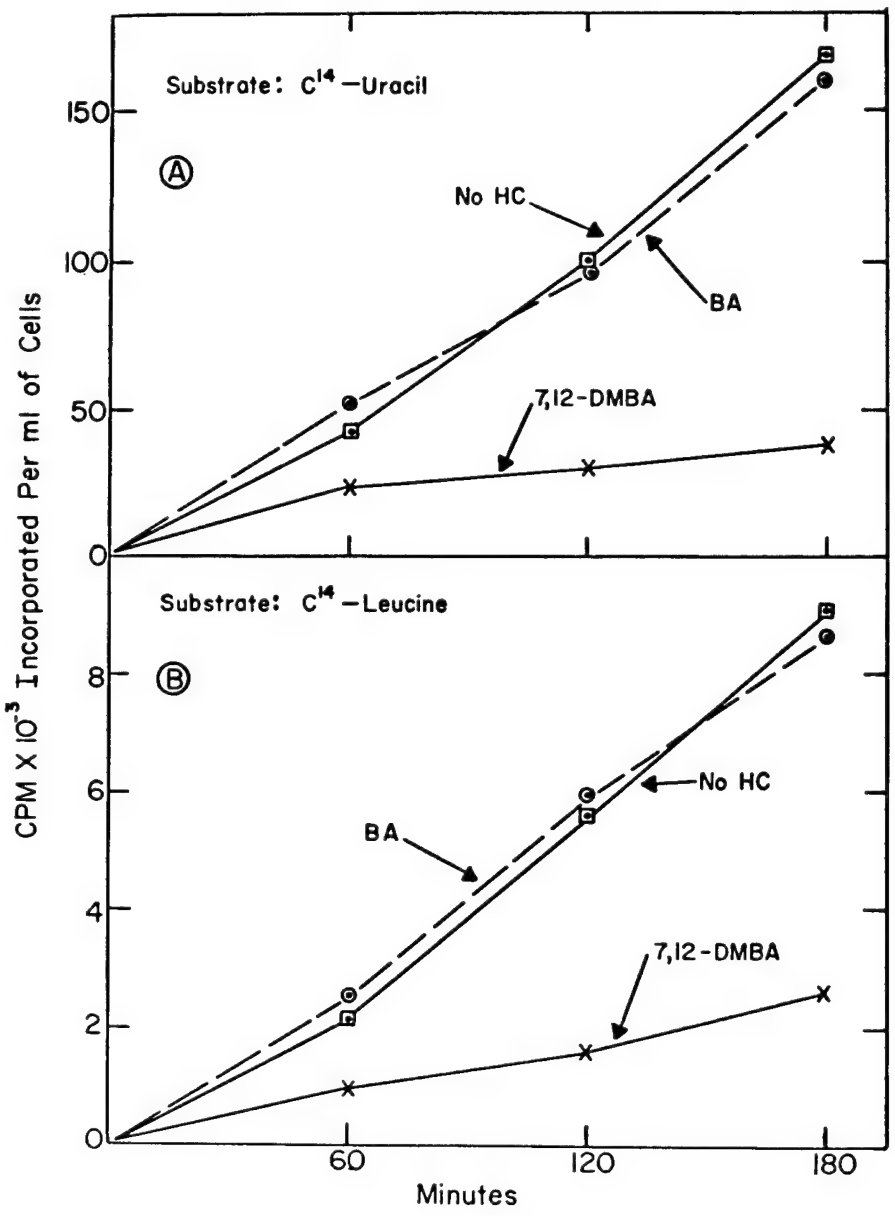

Figure 4. Effect of aromatics on E. coli spheroplast synthesis of RNA and protein. The reaction mixture contained 1 ml of E. coli spheroplasts (1×10^8 cells, "CA method"), 20 μg of hydrocarbon and either 0.01 mc of uracil-2-^{14}C (45 mc per mmole) or 0.01 mc of ^{14}C-leucine (222 mc per mmole). The mixture was incubated at 30° and at various times 0.2 ml samples were withdrawn and the label content of the acid-precipitable fraction determined as described under Method.

e.g., the aromatics 3-aminochrysene, benzo(a)pyrene and 3-methylcholanthrene exhibit stronger inhibitory responses when assayed by the infectious nucleic acid procedure rather than by the "CA method." When spheroplasts infected with the single-stranded viruses are employed, a striking correlation is observed between those aromatics that inhibit viral multiplication and those that induce tumors in rats. The converse correlation for the inactive hydrocarbons is also impressive. Since hydroxylated derivatives of DMBA have been the subject of some attention in

mammalian studies,[10] it might be mentioned that under assay conditions where 7,12-DMBA inhibits MS2 replication by more than 90 per cent, the aromatics 7,12-dihydroxymethyl-BA and 7-hydroxymethyl-12-methyl-BA are without effect, and 12-hydroxymethyl-7-methyl-BA inhibits replication by only 30 per cent. These results are in agreement with the mammalian work, where the hydroxymethyl aromatics were found to be less carcinogenic than DMBA. Although the close relationship between hydrocarbon action in the bacterial and mammalian systems may be fortuitous it is interesting that the viral and mammalian studies show that certain aromatics similarly protect against 7,12-DMBA-induced activity.[8]

Our studies with labeled precursors and MS2 antiserum suggest that hydrocarbon inhibition of MS2 replication involves a reduction in the rate and extent of viral nucleic acid and protein synthesis. The reduced level of viral RNA polymerase in the presence of 7,12-DMBA offers a possible explanation for the lower rate of MS2 RNA synthesis, although the mechanism by which the hydrocarbon reduces this activity cannot be ascertained from these experiments. The net result of the hydrocarbon action appears to be an inhibition in the number of infected cells producing virus, and a lowering of the viral burst size for those cells that do produce virus. It should be pointed out that our assays for de novo virus synthesis detect only those viruses that are infectious; defective viruses would not be scored. Since a large number of infected spheroplasts appear to produce no phage in the presence of active hydrocarbons, the question may be raised whether 7,12-DMBA is lethal to the host cell. Titration experiments with penicillin spheroplasts, which are also sensitive to 7,12-DMBA and which can revert to whole cells after removal of the antibiotic, show no detectable decrease in potential colony formers in the presence of 7,12-DMBA.

In view of our present finding that the active hydrocarbons also reduce the efficiency of the host's biochemical machinery to synthesize nucleotides, nucleic acids and proteins, the viral inhibition might simply be a reflection of the reduced capacity of E. coli cells to manufacture these metabolites in general. Although this explanation is the easiest to accept, it does not account for our results with the double-stranded viruses T4 and λ. If the primary action of the active aromatics is on the host, then the replication of all viruses should be similarly affected; the aromatics inhibitory for MS2 and ϕX174 replication, however, show reduced or little inhibitory activity on T4 and λ replication. DeMaeyer et al. have reported that carcinogenic hydrocarbons inhibit the replication of the double-stranded DNA mammalian viruses, herpes and vaccinia, in rat embryo cultures,[11] yet these workers could demonstrate no inhibition by the same carcinogens on a mammalian RNA-containing virus, Sindbis. Basic differences may exist between the hydrocarbon response of the microbial and mammalian viral systems. At present, our studies offer no explanation for the differences in hydrocarbon response between the single- and double-stranded viruses, but the differences in replication mechanism of these two types of viruses may allow one to observe more definitive effects in one case than in the other. If this were true, it would imply that the active aromatics exert their influence at multiple loci in infected bacteria, at sites sensitive to the operation of the host's metabolic machinery as well as at sites unique to the viral replicative process.

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THE EFFECT OF TRYPSIN ON ESCHERICHIA COLI AND RABBIT RETICULOCYTE RIBOSOMES*

By

R. Zak,† K. G. Nair, and M. Rabinowitz‡

We have previously reported experiments which suggest that ribosomal aggregates from heart muscle are stabilized by interaction between nascent peptide chains.[1] Treatment of the ribosomal assemblage with a proteolytic enzyme, either chymotrypsin or trypsin, as well as with ribonuclease is required to release monomers; neither enzyme alone is completely effective. Similar results have been reported for the chick embryo polysomes that synthesize collagen,[2] and for the ribosomal aggregates isolated from lymph nodes that are active in γ globulin synthesis.[3] Myofibrillar proteins, collagen and γ globulin are all composed of subunits having a strong tendency for spontaneous interaction. It was therefore concluded that an interaction between newly synthesized polypeptide chains might in certain circumstances contribute to the stability of polysomes.

Liver and reticulocyte polysomes, in contrast to those from muscle, connective tissue, and lymph nodes, are completely converted to monomers, or into Mg^{2+}-stabilized dimers, by ribonuclease treatment.[4,5] Moreover, Williamson and Schweet have shown that treatment of ribosomes from reticulocytes with puromycin leads to the release of peptide chains but not to breakdown of the ribosome aggregates unless energy is added.[6] Since mild trypsin treatment of liver[1,7,8] and reticulocyte[8] polysomes also leads to their breakdown, it would appear that proteolytic enzymes affect the ribonucleoprotein complex not only by causing the release of nascent peptide chains, but also by an effect on the structure of the ribosome.

To determine the means whereby proteolytic enzymes alter the state of aggregation of ribosomes, a systematic study of the effect of mild proteolysis on ribosomal structure and function was undertaken. It is the purpose of this paper to report the results of that study. Relatively mild tryptic digestion caused an inhibition of the binding by ribosomes of polyuridylic acid. It is possible that secondary to this effect, the binding of aminoacyl-sRNA to ribosomes and the incorporation of ^{14}C-labeled amino acid into protein were also inhibited. Trypsin also caused a dissociation of E. coli and reticulocyte monomers into their subunits; trypsin-treated subunits failed to recombine to form 70S particles when incubated in the presence of an adequate concentration of magnesium. The results of trypsin treatment of ribosomes and polysomes emphasize the importance of the protein moiety of the ribosome in the binding of artificial, and probably also of natural, messenger RNA, and in the Mg^{2+}-requiring association of ribosomal subunits. Trypsin appears then to disrupt polysomes not only by removal of nascent peptide chains, but also by dis-

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turbing the aminoacyl-sRNA-ribosome-mRNA* complex through damage to the ribosomal protein.

MATERIALS AND METHODS

E. coli strain B was grown in an enriched medium[9] and harvested in the exponential phase of growth. Ribosomes were isolated, pre-incubated and washed as described by Nirenberg and Matthaei.[10] Reticulocytes were obtained from anemic New Zealand albino rabbits by cardiac puncture after treatment of the animals with 2.5 per cent phenylhydrazine. The polyribosomes and monomers were isolated according to the method of Arnstein et al.[11]

Assay of the binding of ^{14}C-phenylalanyl-sRNA to ribosomes was carried out with Millipore membrane filters as described by Nirenberg and Leder.[12] The 70S ribosomes and the 50S subunits adhere quantitatively to the membrane, but approximately 30 per cent of the 30S subunits pass through. Incorporation of ^{14}C-phenylalanine into protein by ribosomes was determined according to the method of Nirenberg and Matthaei;[10] incorporation was terminated by the addition of 5 per cent trichloracetic acid, and 1 mg of bovine serum albumin was added. The specific circumstances of the experiments are given in the tables and figures. The precipitate was washed with cold and hot trichloracetic acid on a Millipore membrane filter. The membranes and the protein were dissolved in Bray's solution and radioactivity was determined with 49 per cent efficiency in a Tricarb Liquid Scintillation Counter.

Trypsin and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation; ^{14}C-labeled and non-radioactive polyuridylic acid were purchased from Miles Chemical Corporation; ^{14}C-L-phenylalanine, having a specific activity of 333 mC/mM, was obtained from New England Nuclear Corporation. E. coli stripped sRNA (General Biochemicals) was charged with ^{14}C-phenylalanine and nineteen ^{12}C-amino acids;[13] the product had a specific activity of 1-1.6 x 10^5 cpm/mg sRNA.

Treatment of ribosomes with 5 μg trypsin† for 30 min at 0° leads to the release of less than 0.3 per cent of 280 mμ absorbing material.

The effect of trypsin on the following ribosomal properties was studied: (1) the dissociation of monomers into subunits; (2) the reassociation of subunits; (3) the binding and release of ^{14}C-polyuridylic acid; (4) the binding and release of ^{14}C-phenylalanyl-sRNA; (5) the incorporation of ^{14}C-phenylalanine into protein. In each of the tests the effect of trypsin was completely blocked by the prior addition of a threefold excess of soybean trypsin inhibitor. Neither trypsin inhabitor, nor DFP trypsin caused a change in ribosome function. No ribonuclease activity was detectable in the trypsin.

RESULTS

(1) Dissociation of monomers into subunits. There was no appreciable breakdown of E. coli ribosomes to 50S and 30S subunits when treated for 30 min at 0° with 0.5-5 μg of trypsin (Figure 1A), or for 5 min at 25° (Figure 2). When 10 μg of trypsin was used, however, the 70S peak was

*The following abbreviations are used: sRNA - soluble ribonucleic acid, mRNA - messenger ribonucleic acid, Phe - Phenylalanine, DFP - diisopropylfluorophosphate, poly U - polyuridylic acid.

†In each case the concentration of trypsin is in μg of enzyme per optical density unit (at 260 mμ) of ribosomes.

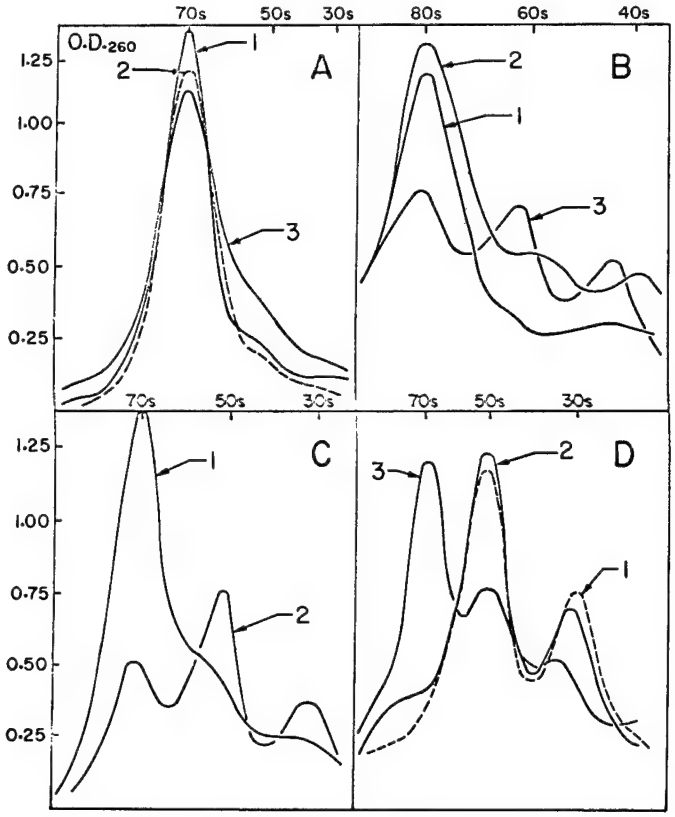

Figure 1. Sucrose gradient centrifugation of ribosomes treated with trypsin. The concentration of ribosomes is given as the absorption at 260 mμ. A 0.1 ml aliquot of ribosomes containing 2-3 O.D.$_{260\ m\mu}$ was analyzed on a 5-20 per cent linear sucrose gradient prepared with the same buffer as that in which the ribosomes were suspended. The gradient was centrifuged at 39,000 rpm in the SW 39 rotor of the model L2 Spinco centrifuge for 90 min at 0°. The sedimentation profiles were recorded with a Gilford spectrophotometer, model 2000. Ribosomes or ribosomal subunits were exposed to trypsin for 30 min at 0°, following which soybean inhibitor was added in 3-fold excess.

A. E. coli ribosomes: curve 1 - control; curve 2 - treated with 0.5 μg trypsin/O.D.$_{260\ m\mu}$; curve 3 - treated with 10 μg trypsin/O.D.$_{260\ m\mu}$.

B. Reticulocyte monomers purified by differential centrifugation; curve 1 - control; curve 2 - treated with 0.7 μg trypsin/O.D.$_{260\ m\mu}$; curve 3 - treated with 10 μg trypsin/O.D.$_{260\ m\mu}$.

C. Dissociated and reassociated E. coli ribosomes. Ribosomes were dissociated by dialysis for 3 h against 5×10^{-3}M Tris-HCl buffer pH 7.3, 10^{-4}M $MgCl_2$; and were reassociated by overnight dialysis against 5×10^{-3}M Tris-HCl buffer pH 7.3, 1.75×10^{-2}M $MgCl_2$; 8.6×10^{-2}M KCl, and 6×10^{-3}M mercaptoethanol. Trypsin treatment followed reassociation. Curve 1 - no trypsin treatment; curve 2 - treated with 5 μg trypsin/O.D.$_{260\ m\mu}$.

D. Effect of tryptic treatment on reassociation of 50S and 30S subunits. Curve 1 - E. coli ribosomes dissociated into 50S and 30S subunits by dialysis against low Mg^{2+}; curve 2 - 50S and 30S subunits treated with 5 μg trypsin/O.D.$_{260\ m\mu}$ followed by reassociation; curve 3 - 70S ribosomes treated with 5 μg trypsin/O.D.$_{260\ m\mu}$ followed by dissociation and reassociation.

somewhat decreased in height and broadened, indicating slight breakdown of 70S particles (Figure 1A, curve 3). On rare occasions a ribosomal preparation was unusually sensitive to trypsin and complete disruption into 50S and 30S subunits occurred with 2 μg trypsin.

The "trypsin resistant" ribosomes could be transformed into "sensitive" ones by dissociating them to subunits in 10^{-4}M Mg^{2+}, and then reassociating the subunits in 10^{-2}M Mg^{2+}. The reconstituted ribosomes were extremely sensitive to trypsin, as little as 0.5 μg of enzyme now causing their dissociation (Figure 1C). The reason for this is not clear; it may have been the result of the release of a ribosomal component (mRNA, sRNA or the nascent peptide chain) during dissociation; or the subunits may not reassociate in a manner identical to their original structure.

(2) Reassociation of subunits. Trypsin-treated ribosomes may be dissociated by dialysis against a low concentration of Mg^{2+}, and reassociated to 70S particles by dialysis against buffer containing 10^{-2}M Mg^{2+} (Figure 1D, curve 3). The reassociation was however, 20-30 per cent less complete when the ribosomes had been treated with trypsin (compare Figure 1D, curve 3 with Figure 1C, curve 1). If, instead of the 70S monomers the subunits obtained by dialysis were exposed to trypsin, no reassociation occurred (Figure 1D, curve 2). The subunits were far more sensitive to tryptic digestion than the intact ribosomes, perhaps because the association to form 70S monomers partly protects the sites required for cohesion of the subunits.

Similarly, 70S ribosomes isolated from Strep. pyogenes[14] and liver[15] are relatively resistant to ribonuclease, but become sensitive to the enzyme when dissociated to their subunits.

Reticulocyte ribosomes react to trypsin treatment in a manner similar to that of E. coli ribosomes except that they are more sensitive to the enzyme. As little as 0.7 μg of trypsin at 0° for 30 min causes considerable increase in the numbers of 60S and 40S particles (Figure 1B). Yeast ribosomes also are split into subunits by treatment with papain for 12 min at 37°.[16]

(3) Binding and release of ^{14}C-polyuridylic acid. The binding of ^{14}C-polyuridylic acid to E. coli 70S ribosomes was decreased by 75 per cent after treatment with 5 μg of trypsin for 5 min at 25° (Figure 2). The results accord with those of Kaji and Kaji.[17] The ^{14}C-polyuridylic acid bound to 70S ribosomes by incubation for 20 min at 0° was completely released by treatment with trypsin. The amount of ^{14}C-polyuridylic acid bound to E. coli ribosomes was not affected by the presence of aminoacyl sRNA. Furthermore, the addition of aminoacyl sRNA (20 μg sRNA per O.D.$_{260}$) did not prevent the trypsin-induced release of polyuridylic acid from the ribosome. Trypsin did not alter the sedimentation (7S) of ^{14}C-polyuridylic acid in a linear sucrose density gradient.

(4) Binding and release of ^{14}C-phenylalanyl sRNA. Treatment of 70S ribosomes from E. coli with 0.5, 5, or 10 μg of trypsin caused a decrease in the polyuridylic acid directed binding of ^{14}C-phenylalanyl-sRNA to the ribonucleoprotein particles (Table 1 and Figure 2A). Analysis of the ribosomes on sucrose gradients disclosed that the decrease in binding of ^{14}C-phenylalanine-sRNA occurred even though less than 10 per cent of the ribonucleoprotein particles were split to subunits. Ribosomes from E. coli and reticulocyte were treated with trypsin and centrifuged in a sucrose gradient; the 70S particles isolated from the gradient showed a reduction of ^{14}C-phenylalanyl-sRNA binding similar to that of the whole preparation. Thus the inhibition by trypsin of the binding of aminoacyl-sRNA to ribosomes appears not to be solely a consequence of the formation of subunits.

When E. coli ribosomes to which aminoacyl-sRNA was bound were treated with 0.5, or 5.0

Table 1

EFFECT OF TRYPSIN ON BINDING AND INCORPORATION OF ^{14}C-PHENYLALANYL-sRNA BY E. coli AND RABBIT RETICULOCYTE RIBOSOMES

Experiment	E. coli			Rabbit reticulocytes					
				Monomers			Polysomes		
	Control (cpm)	Trypsin (cpm)	Decrease (%)	Control (cpm)	Trypsin (cpm)	Decrease (%)	Control (cpm)	Trypsin (cpm)	Decrease (%)
^{14}C-PHENYLALANYL-sRNA									
Binding	981	578	42	1703	598	65			
Release	665	639	4	332	199	40			
INCORPORATION OF ^{14}C-PHENYLALANINE									
Without poly U	139	117	21	190	139	27	2460	980	60
With poly U	5126	527	90	5442	685	88	5417	1006	82

E. coli ribosomes were preincubated for 45 min at 35° with the complete system for assaying amino acid incorporation into protein.[10] The medium (Medium I) used to measure ^{14}C-phenylalanyl-sRNA binding to ribosomes contained in 0.1 ml: 2-4 x 10^3 cpm ^{14}C-phenylalanyl-sRNA; 10 μmoles Tris-HCl buffer pH 7.6; 2 μmoles magnesium acetate; 8 μmoles NH_4Cl; and 20 μg polyuridylic acid. Incubation was for 15 min at 35°. The medium (Medium II) used to assay ^{14}C-phenylalanine incorporation into protein by E. coli ribosomes contained in 0.1 ml: 5 x 10^4 cpm ^{14}C-phenylalanine; 5 μmoles Tris-HCl buffer pH 7.6; 1.5 μmoles $MgCl_2$; 5 μmoles KCl; 0.75 μmoles mercaptoethanol; 0.1 μmole ATP; 0.2 μmole phosphoenolpyruvate; 0.025 μmoles GTP; 2.5 μg pyruvate kinase; 0.1 mg E. coli 105,000 x g supernatant protein; and 20 μg polyuridylic acid when indicated. For ^{14}C-phenylalanine incorporation into protein by reticulocyte ribosomes, the system was the same except that the $MgCl_2$ concentration was 8mM; 10mM glutathione was used in place of the mercaptoethanol; and 1 mg of reticulocyte 'pH 5 enzymes' was used. Incubation was for 15 min at 35° when ribosomal monomers were used and 5 min at 35° when polysomes were employed. In each assay, approximately 2 O.D.$_{260\ m\mu}$ of ribosomes were present. Results are expressed as cpm of ^{14}C-phenylalanine bound or incorporated per O.D.$_{260\ m\mu}$ of ribosomes in 30 min at 0°. To test for release of bound ^{14}C-phenylalanyl-sRNA, trypsin treatment followed incubation with ^{14}C-phenylalanyl-sRNA. The values are the average of duplicate determinations.

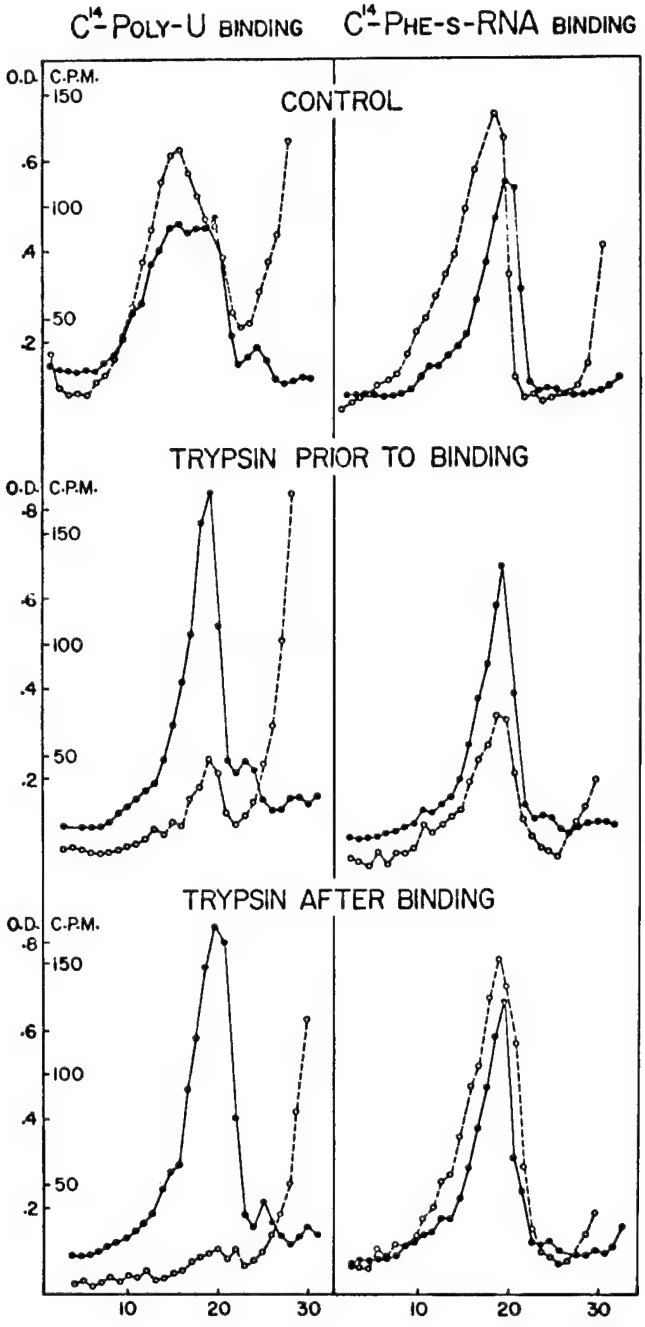

Figure 2. Effect of trypsin on the binding of ^{14}C-polyuridylic acid and ^{14}C-phenylalanine-sRNA to E. coli ribosomes. The ribosomes were prepared as described in Table 1. The conditions for assay of the binding of ^{14}C-polyuridylic acid to ribosomes were identical to those of Suarez and Nathans.[18] The 0.2 ml reaction mixture contained 2 x 10^4 cpm of ^{14}C-polyuridylic acid (specific activity 0.183 μC/mg), and 7 O.D.$_{260\ m\mu}$ of ribosomes. The conditions for measuring the binding of ^{14}C-phenylalanyl-sRNA to ribosome were described in Table 1. Ribosomes were treated with 5 μg of trypsin per O.D.$_{260\ m\mu}$ for 5 min at 25°, and 15 μg of trypsin inhibitor was then added. Density gradient centrifugation was carried out as described in Figure 1. The tubes were punctured and 10 drop fractions were collected and processed as described before.[1] ● — ● optical density at 260 mμ; o - - - - o cpm.

μg of trypsin, no release of radioactivity occurred (Table 1; Figure 2).

Trypsin decreased the amount of ^{14}C-phenylalanyl-sRNA bound to reticulocyte monomers, as was true for E. coli ribosomes (Table 1). About 40 per cent of the ^{14}C-phenylalanyl-sRNA bound to reticulocyte ribosomes was removed by subsequent treatment with 5 μg trypsin in contrast to the results obtained with E. coli ribosomes (Table 1).

The effect of trypsin on ^{14}C-phenylalanyl-sRNA binding and upon ^{14}C-polyuridylic acid binding to ribosomes was not of the same degree. Although the binding of charged sRNA required polyuridylic acid, trypsin doses which result in more than an 80 per cent reduction of polyuridylic acid binding led to only a 50 per cent decrease in sRNA binding. Moreover, trypsin caused the release of all the polyuridylic acid bound to ribosomes from E. coli, whereas little or no charged sRNA was liberated. The results may be accounted for by transfer of the aminoacyl sRNA from a ribosomal site requiring polyuridylic acid to another site where it is bound directly to the ribosome. A binding site of this type has been suggested by Nirenberg and Leder.[12]

(5) Incorporation of ^{14}C-phenylalanine into protein. Incorporation of ^{14}C-phenylalanine into protein by either E. coli or reticulocyte ribosomes was greatly impaired by trypsin treatment (Table 1). The stimulation by polyuridylic acid of phenylalanine incorporation into protein by ribosomal monomers was nearly abolished by trypsin as might have been predicted from the fact that the enzyme effected a decrease in the binding of polyuridylic acid. The incorporation of ^{14}C-phenylalanine into reticulocyte polysomes containing natural messenger RNA is affected to a lesser degree (Table 1). Trypsin had less effect on the incorporation of ^{14}C-phenylalanine into monomers in the absence of polyuridylic acid. It would appear that the attachment of natural messenger to ribosomes is more resistant to trypsin than is polyuridylic acid. Trypsin was still effective when ^{14}C-phenylalanyl-sRNA and polyuridylic acid were added prior to the proteolytic enzyme (Table 2).

DISCUSSION

The experiments indicate that the binding of polyuridylic acid to ribosomes is markedly diminished by alteration or removal of one or more ribosomal peptides. The proteolytic treatment was mild, as indicated by the unchanged sedimentation constant and the absorption ratio (280 mμ: 260 mμ) of the monomers. To what extent the ribosome protein participates in the binding of polyuridylic acid and natural messenger RNA is not known. It is possible that a specific configuration or structure of the ribosomal protein is required for the binding of messenger RNA to the ribosomal RNA. Had the prior association of polyuridylic acid and sRNA with the ribosome protected against proteolytic destruction of the binding site, this possibility would have been reinforced.

The reduction of ^{14}C-phenylalanyl-sRNA binding to ribosomes by trypsin was of smaller magnitude than, and may well be secondary to the inhibition of polyuridylic acid binding. The binding of that aminoacyl sRNA to ribosomes is dependent on the presence of natural or artificial messenger RNA. The release of template RNA from the ribosome could then secondarily cause the loss of aminoacyl sRNA. The observation that ^{14}C-phenylalanyl sRNA bound to ribosomes in the presence of polyuridylic acid is not released by tryptic digestion despite the release of bound polyuridylic acid is difficult to explain. The aminoacyl sRNA may be transferred to another binding site which is not dependent on the presence of polyuridylic acid. Although the

Table 2

COMPARISON OF THE ABILITY OF E. coli RIBOSOMES TO SYNTHESIZE ^{14}C-POLYPHENYLALANINE AFTER TRYPTIC TREATMENT IN THE PRESENCE OR ABSENCE OF PREVIOUSLY ADDED ^{14}C-PHENYLANANYL-sRNA AND POLY U

System	Cpm/O.D.$_{260}$	Decrease (%)
Ribosomes (control)	2148	
Ribosomes pre-treated with trypsin, then ^{14}C-Phe-sRNA and poly U added	1101	51.5
Ribosomes (control)	1930	
Ribosomes incubated with ^{14}C-Phe-sRNA and poly U first, then trypsin treated	915	52.5

^{14}C-phenylalanyl-sRNA and polyuridylic acid were bound to E. coli ribosomes either before or after trypsin treatment as indicated. The incubation conditions for binding were as in Table 1. Trypsin was added at a concentration of 0.5 μg/O.D.$_{260}$ ribosome and the reaction terminated, after incubating for 30 min in ice, by a threefold excess of soybean trypsin inhibitor.

Ribosomes thus pretreated were incubated for a second time in a mixture containing energy and 105,000 g supernatant enzymes (Medium II, Table 1) for 10 min at 35°.

effect of trypsin on phenylalanyl-sRNA binding can be adequately explained as secondary to a decrease in polyuridylic acid binding, an additional effect on ribosomal structure to alter an aminoacyl RNA binding site cannot be ruled out.

Similarly the inhibition of ^{14}C-phenylalanine incorporation into protein by trypsin could be secondary to the effect of the enzyme on the binding and release of polyuridylic acid. If so, the inhibition by trypsin of amino acid incorporation into protein observed with reticulocyte polysomes, and with E. coli and reticulocyte monomers, suggests that natural messenger RNA is also released by proteolytic action. The polyuridylic acid directed amino acid incorporation of ^{14}C-phenylalanine, was in all cases more sensitive to tryptic action than incorporation directed by natural messenger. Thus natural messenger RNA bound to ribosomes may be more resistant to release by trypsin than is bound polyuridylic acid. It is possible that this is an example of more effective protection of those binding sites from tryptic digestion by the bound messenger RNA than by polyuridylic acid.

An additional site of action of tryptic digestion is required to account for the enhanced formation of subunits from E. coli ribosomes. It also appears that the peptide configuration required for this Mg^{2+}-linked association of subunits is exposed when ribosomes are dissociated into 50S and 30S subunits. These subunits are much more sensitive to tryptic digestion than are 70S ribosomes.

The disruption of polyribosomes from E. coli or reticulocytes by proteolytic enzymes would seem then to be the result of an alteration of the binding site for messenger RNA; the messenger RNA is released and the ribosomal assemblage breaks down. Inasmuch as complete dissociation of E. coli and reticulocyte ribosomes was obtained by ribonuclease, it is unnecessary to postulate an interaction between nascent polypeptide chains for the stabilization of the

polyribosomes. The failure of proteolytic enzymes to affect the polysomal structure from rabbit skeletal muscle reported by Breuer et al.[19] may be due to a relative insensitivity of the messenger binding site in that preparation.

However, where ribonuclease does not completely disrupt the polyribosomal structure, as in the case of the ribosomes concerned with the synthesis of myofibrillar protein, collagen or γ globulin, it is possible that an interaction between ribosomal-bound polypeptide chains containing the subunits of the protein participates in stabilization of the structure.

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MODIFICATION OF RADIATION RESPONSE OF TISSUE BY ACTINOMYCIN - PRELIMINARY CLINICAL EVALUATION*

By

M. L. Griem, and K. Ranninger†

One of the problems of the radiotherapist is that he must deal with malignant tumors that are resistant to irradiation at dose levels which can be tolerated by the normal surrounding tissues. It is therefore desirable to increase the radiosensitivity of the tumor, or to potentiate the effects of radiation within the tumor without producing a proportional increase in the radiosensitivity of the normal tissue.

A number of reports have suggested that the drug actinomycin might enhance the radiosensitivity of certain tumors that are normally resistant to radiation therapy.[1-6] Our initial experimental studies were conducted on the effects of combining injections of actinomycin D with radiation on a solid transplantable Walker 256 tumor in rats.[7] Particular attention was given in these studies to the parameter of the time interval intervening between injection of the drug and delivery of radiation. Rats bearing Walker 256 carcinoma-sarcoma in the hind leg were treated when the tumor measured approximately 1 cm in diameter. One hundred fifty mg/kg of actinomycin D‡ was injected intraperitoneally 24 hours, 4 hours, and 1 hour before local irradiation of the tumor with a single dose of 2500 r air (250 KVP x rays, HVL 1.5 mm Cu, F.S.D. 50 cm). Controls consisted of untreated tumor animals, drug-treated animals, and x-ray treated animals. The most rapid tumor growth and the shortest survival times were seen when the actinomycin was given 24 hours before irradiation.

These studies were extended to observe the effects of actinomycin and radiation upon mice, using a microscopic hair-indicator system to evaluate the extent of cellular damage to the anagen hair matrix induced by this combined treatment, again with particular reference to the relationship between times of drug injection and irradiation.[8]

Subsequently, patients with advanced malignancies were studied using the drug in conjunction with the usual palliative therapeutic doses of x-rays. Adult patients were given intravenous injections of 0.5 mg of actinomycin D followed after 3 to 4 hours by a 500 rad minimum dose to the tumor. This sequence was repeated twice weekly for 2 to 4 weeks (total cumulative dose 2,000 to 4,000 rads).

In an attempt to make comparative evaluations of the effects of the drug alone, of radiation alone, and of combined drug-radiation treatment, patients with round metastases to both lungs were selected for clinical trials. In one group of these patients treatments were given in the following sequence: (1) x-ray therapy to one lung field alone; followed after some weeks by (2) drug and radiation to the opposite lung field; and (3) drug therapy to the lung previously irradiated in

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(1). In the other group of patients the sequence was reversed, being: (1) drug alone to lung No. 1; (2) drug and x-ray to lung No. 2; (3) x-ray to lung No. 1. Evaluation of cases was made by the following criteria:

A. All lesions were histologically proven before treatment was started;
B. Lesions were compared at autopsy or on the basis of histologic evidence; or
C. Objective measurements of the lesions were made on x-ray 2 to 6 months after therapy;
D. Comparison of the effects of drug or x-ray alone, or in combined treatment: Where multiple lesions were available for study, the same fractionation and dose of x-rays were used; where multiple lesions were not available, a total x-ray dose known to be well below the tumoricidal range was used.
E. Subjective improvement was not considered.

Tables 1 and 2 show the types of tumors treated and those that showed objective clinical response.

Table 1

ACTINOMYCIN AND RADIATION

53 cases - total series	
11 cases excluded because	
a. lost to follow-up	
b. expired soon after treatment	
c. recently treated	
42 cases evaluated	
9 cases	no response to any treatment (-)
10 cases	response which could be due to x-ray alone or combined treatment (+)
23 cases	response due to combined treatment (++)

DISCUSSION

In making a clinical evaluation of the combined use of drug and irradiation, it is difficult to decide whether the effects produced are merely additive, or whether there is a true therapeutic synergism resulting in an enhanced response in the irradiated tumor compared to the response in the normal tissues of the host. We are continuing our investigations into the clinical aspects of this problem, and in addition are studying the effects of similar treatments on other animal tumors in an attempt to clarify the matter further.

The mechanism of the joint action of actinomycin and radiation might be explained in two ways. The basis for the observed cyclic response might lie in the observations of Hackmann[9] who found that in mice, a dose of 2 to 5 micrograms of actinomycin produced an increase in the number of mitoses between 1 and 5 hours after administration of the drug. During the subsequent 17-hour period the number of mitoses decreased and had returned to normal by 22 hours after the time of injection. Tolmach[10] has shown that there are variations in radiosensitivity during the cell cycle, the maximum sensitivity being reached during mitosis. It is therefore possible that the maximum effect of drug and irradiation is produced when irradiation of the tumor coincides with the period of radiosensitivity induced by drug administration.

Table 2

ACTINOMYCIN AND RADIATION

No. of cases	Histology	Response
2	Osteosarcoma	2-
2	Angiosarcoma	1+ 1++
2	Rhabdomyosarcoma	2++
3	Myosarcoma	3++
2	Neurogenic sarcoma	2++
1	Leiomyosarcoma	1++
7	Multiple myeloma	4++ 2+ 1-
1	Hemangioendothelioma	1++
2	Neuroblastoma	1- 1+
2	Malignant meningioma	1++ 1+
1	Malignant mesothelioma	1++
1	Osteochondrosarcoma	1++
1	Undifferentiated sarcoma of orbit	1+
1	Liposarcoma	1-
1	Mixed mesodermal sarcoma	1-
2	Melanoma	1++ 1-
3	Wilm's tumor	2+ 1++
1	Muco-epidermoid carcinoma of parotid	1++
3	Squamous cell carcinoma	
	skin	1+++
	lip	1+++
	floor of mouth	1-
3	Adenocarcinoma of ovary	2++ 1+
1	Embryonal cell carcinoma	1+

A second explanation might be based on the observations of Kaplan and co-workers[11] who have demonstrated that the radiosensitivity of a cell varies with the content of guanine in the nucleus. Goldberg and co-workers[12] have shown that the action of actinomycin is to inhibit DNA dependent protein synthesis within the cell by attaching to the guanine residue.

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MODIFICATION OF RADIATION RESPONSE OF TISSUE BY COLCHICINE: A CLINICAL EVALUATION*

By

M. L. Griem† and F. D. Malkinson‡

In a previous paper,[1] we published our findings on a preliminary evaluation of the drug colchicine as a modifier of the radiation response of tissues. This study reported a series of fifty patients with advanced malignancies that were considered inoperable and unsuited for routine palliative x-ray therapy. Preliminary experiments with colchicine and x-rays, using the rodent hair system and solid mouse transplantable tumors as indicators, suggested that the best treatment effects could be obtained when the drug was injected 16 hours before exposure to x-rays. Since that time, 89 more patients have been treated, and it seems important that the results of these treatments should be published.

The treatment schedule was as follows: In most patients, 4 to 5 milligrams of colchicine were given intravenously, followed by a minimum x-ray dose of 500 rads delivered to the tumor. This sequence was repeated every fourth or fifth day until a total tumor dose of 4,000 rads had been delivered. In evaluating response to this treatment, the criteria used were those described in the preceding paper in this report by Griem and Ranninger on the effects of radiation and actinomycin.[2]

Table 1 shows the number of cases in the study, the number of autopsies, the number of patients dead but not autopsied, and the number of patients alive. When there was no autopsy, some of the lesions had been biopsied or removed surgically for histological evaluation following treatment.

Table 1

CENSUS

Total cases	138
Autopsies	65
Dead, but no autopsy	61
Alive	12

Table 2 shows the method of evaluation and the number of cases in each of the groups.

In Table 3, the histology of the type of lesion evaluated and the location of the lesion are given, together with the number of cases and the evaluation of the response in those cases. An

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Table 2

CRITERIA FOR EVALUATION

Insufficient time for evaluation or lost to follow-up	23 (0)
No response to drug, radiation or combined treatment	24 (-)
Response to radiation without evidence of potentiation from colchicine	22 (*)
Patients showing objective response to combination of colchicine and radiation	69 (**)

Table 3

HISTOLOGICAL EVALUATION

Adenocarcinoma of esophagus	4 cases			2 (*)	2 (**)
Squamous cell ca esophagus	1 case		1 (-)		
Adenocarcinoma of stomach	22 cases	3 (0)		4 (*)	15 (**)
Liver metastases treated	3 cases	1 (0)		1 (*)	1 (**)
Adenocarcinoma of duodenum	1 case				1 (**)
Adenocarcinoma of colon	31 cases	4 (0)	1 (-)	2 (*)	24 (**)
Liver metastases treated	4 cases	2 (0)	1 (-)		1 (**)
Lung metastases treated	3 cases		1 (-)	1 (*)	1 (**)
Bone metastases treated	2 cases	1 (0)		1 (*)	
Skin metastases treated	1 case	1 (0)			
Adenocarcinoma of pancreas	11 cases	5 (0)	1 (-)	3 (*)	2 (**)
Liver metastases treated	1 case		1 (-)		
Lung metastases treated	1 case	1 (0)			
Adenocarcinoma of gallbladder	2 cases			1 (*)	1 (**)
Hepatocarcinoma	1 case		1 (-)		
Adenocarcinoma of lung	7 cases		2 (-)	1 (*)	4 (**)
Undifferentiated ca lung	8 cases	3 (0)	2 (-)	1 (*)	2 (**)
Squamous cell on lung	5 cases	1 (0)	2 (-)	1 (*)	1 (**)
Hypernephroma	5 cases		5 (-)		
Melanoma	10 cases		4 (-)	2 (*)	4 (**)
Mycosis fungoides	5 cases		1 (-)		4 (**)
Adenocarcinoma of prostate	1 case	1 (0)			
Transitional cell ca bladder	2 cases		1 (-)		1 (**)
Adenocarcinoma of thyroid	1 case				1 (**)
Adenocarcinoma Gardner's Duct	2 cases			1 (*)	1 (**)
Undifferentiated ca ovary	1 case				1 (**)
Squamous cell ca skin	1 case				1 (**)
Neurogenic sarcoma	1 case				1 (**)
Adenocarcinoma urachal origin	1 case			1 (*)	

impressive response was observed in patients with adenocarcinoma of the colon, and adenocarcinoma of the stomach. In both these tumors, the response in the primary lesion was rather dramatic, but treatment of the metastases was somewhat disappointing. Several patients have lived more than 3 years since treatment of the primary lesions. At autopsy of one patient with adenocarcinoma of the stomach, only necrosis could be found at the site of the tumor. In several other surgical and autopsy specimens, the only evidence of tumor was in the perineural area.

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REDUCED GROWTH RATES OF HAIR IN MICE FOLLOWING EXPOSURE TO SINGLE DOSES OF X-RAY RADIATION*

By

F. D. Malkinson,† and M. L. Griem‡

The proliferative rate of anagen hair matrix cells in the mouse is high, each cell dividing every 12 to 13 hours.[1,2] Such mitotic activity is comparable to that observed in bone marrow stem cells or in intestinal tract epithelium. As with these other cell types, then, the hair matrix cells should provide a highly sensitive indicator system for in vivo studies on the effects of ionizing radiation or of pharmacologic agents, which might yield information useful for the evaluation of cellular responses in other tissues including even malignant tumors. Some investigations exploring these possibilities have recently been reviewed.[3]

The current study was undertaken to observe the effects of single, acute doses of ionizing radiation on the growth rate of anagen mouse hairs, and to compare this effect with that of single exposures on non-proliferating telogen hair matrix cells. We were particularly interested in patterns of radiation injury and recovery that might indicate the usefulness of the hair matrix for microscopic studies of the kinetics of cell depletion and renewal following various forms of tissue injury.

METHOD

Carworth Farms #1 female mice three to six months old were used in these experiments. All animals were plucked on the back above both haunches and then observed for a period of 24 days. Since anagen lasts for 17-20 days in this strain of mouse, preliminary plucking and "cycling" ensured the presence of telogen hairs when subsequent laboratory procedures were begun.

Animals to be irradiated during the anagen phase of growth were plucked once again over the same sites on the 24th day, and a single dose of radiation was given to the plucked site on the right side of the back 10 days later. Hair growth was allowed to continue until the onset of telogen, following which hairs from both the irradiated and control sides of each mouse were plucked for study on the 24th day (14 days after irradiation). Animals treated during telogen received radiation to the right side of the back on the 24th day after the initial plucking, following which both control and irradiated sites were immediately plucked again. Twenty-four days later this "second generation" hair was removed from irradiated and control sites for study.

Radiation was administered with a Machlett OEG-60 tube operated at 50 kV and 30 ma with 2 mm of aluminum added filtration. This produced a surface dose of 387 rads per minute at a focal skin distance of 11 cm and a beam quality of 1.2 cm half value depth in tissue. Single sur-

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face doses of 200 rads, 400 rads, 600 rads, and 800 rads were administered once to separate groups of animals in the anagen hair study. Surface doses of 500 rads, 1000 rads, and 1500 rads were given once to single groups of mice in the telogen study. The animals were adequately shielded and treated individually without anesthesia.

Several hundred hairs from each final sample were floated on a shallow layer of water in a glass Petri dish. The hairs were examined with a binocular dissecting microscope which permitted ready identification of the four hair types of the mouse (Figure 1).[4] Among the anagen irradiated hairs it was often possible to detect microscopic signs of damage such as thinning and

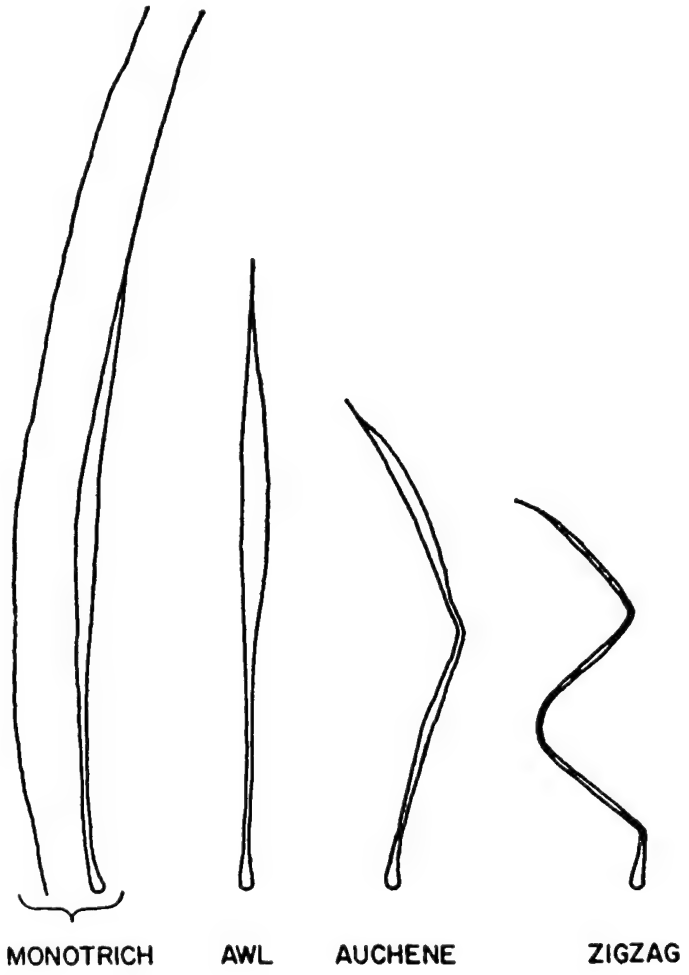

Figure 1.

and irregularity of a portion of the hair shaft. At dose levels of 400 rads and higher most anagen hairs showed varying degrees of such damage, but in each experimental group almost all hairs selected for examination were injured hairs. No such alterations were visible in hairs from the telogen irradiated animals. Twenty to twenty-five intact awl hairs were removed from the Petri dish with an ophthalmological forceps and placed parallel to each other in several rows on a standard 3.25 x 4 inch glass lantern-slide. A 4 x 1 cm strip of translucent graph paper was then placed along one edge of this plate. A second glass plate of the same size was taped firmly

to the first, and the resulting slide was projected on a screen giving a 15-17-fold image enlargement.[5]

The magnified images of all intact hairs were "traced" with pencil on paper, placed over the screen, and their lengths subsequently measured with a flexible rule. Actual hair lengths were calculated by correcting the measured magnified lengths according to the magnification factor of the projected unit scale.

In normal unirradiated animals variations in overall hair length between contralateral sites were less than 2 per cent, and usually under 1 per cent.

Statistical analysis of experimental data included computation of the standard deviation of the mean, standard error of the mean, and 19/20 (95 per cent) confidence limits.

RESULTS

The results of these studies are presented in Tables 1 and 2. The rate of hair growth (as measured by overall hair length) was significantly reduced by a single dose of radiation in all groups of animals examined. In the animals treated during anagen the reduction in hair length after irradiation with 800 rads (13.4 per cent) was significantly greater than that sustained by animals receiving 200 rads (8.3 per cent), establishing a dose-dependent response for these two

Table 1

RETARDATION OF HAIR GROWTH IN MICE IRRADIATED ON THE 10th DAY OF ANAGEN

(Control hairs were plucked from the left flank and irradiated hairs were plucked from the right flank of the same animal)

Dose (rads)	Animals	Average length control (mm)	Average length irradiated (mm)	Per cent growth retardation	Standard error of mean % retardation
200	10	7.92	7.26	8.3	1.53
400	16	7.76	6.85	11.7	0.795
600	11	7.81	6.90	11.7	1.58
800	7	7.70	6.69	13.4	1.11

Table 2

RETARDATION OF HAIR GROWTH OF MICE IN THE SUBSEQUENT GENERATION FOLLOWING IRRADIATION TO THE PRECEDING TELOGEN PHASE

(Control hairs were plucked from the left flank and irradiated hairs were plucked from the right flank of the same animal)

Dose (rads)	Animals	Average length control (mm)	Average length irradiated (mm)	Per cent growth retardation	Standard error of mean % retardation
500	10	7.92	7.52	5.1	0.710
1000	11	8.05	7.03	12.7	0.889
1500	10	7.80	6.88	11.8	0.564

dosage levels. The difference between these two groups may be even greater, since some diffuse alopecia occurred postirradiation and prior to plucking in the animals receiving 800 rads. Treated hairs from this group of animals, then, were essentially "population survivors," and growth impairment may have been greater in the missing hairs.

It is apparent from the tables that there is no difference in the reduced growth rates of the animals treated with 400 rads and those receiving 600 rads (11.7 per cent for both groups). Growth retardation in the hairs from these two groups of animals is probably less than in the animals treated with 800 rads and greater than in those treated with 200 rads, as the actual figures suggest. A precise intermediate position for these figures in the dose response curve cannot be statistically established, however.

Reduction of growth rates induced in anagen hair by irradiation of the preceding generation of telogen hair was similar in animals receiving 1000 rads and 1500 rads (12.7 per cent and 11.8 per cent), and significantly greater than in animals treated with 500 rads (5.1 per cent). Growth retardation for 400-600 rads delivered in anagen was essentially the same as for 1000-1500 rads delivered in telogen. Growth reduction after 200 rads administered in anagen was somewhat greater (8.3 per cent) than for 500 rads given in telogen (5.1 per cent). Only damaged anagen hairs were selected for study in the 200 rad dose range, however, although approximately 50 per cent of the hairs showed no structural abnormality. Since the incidence of significant hair damage may be similar after 200 rads in anagen and 500 rads in telogen, and since hairs plucked after telogen radiation showed no morphologic injury, the selection of many hairs which had sustained little or no damage may have revealed a lessened overall rate of growth reduction in the telogen radiated hairs. The dose response effects observed both in the irradiated anagen and in the irradiated telogen hairs suggest that an approximately 2-1/2 fold greater dose of x-rays must be administered to telogen hairs to produce the same subsequent growth retardation as is required for anagen hair. These conclusions are in close agreement with comparative data for dose responsiveness of anagen and telogen hairs obtained with other experimental techniques in our laboratories.[6]

DISCUSSION

Morphologic abnormalities and depigmentation of hair following irradiation have been widely studied.[7,8] Temporary epilation can be induced by x-rays in anagen follicles[8] and larger doses may produce alopecia in telogen follicles as well.[9] Impaired incorporation of isotope-labeled amino acids and other compounds may follow irradiation.[3] The present study indicates that growth rates of hair may also be reduced by ionizing radiation, and that the degree of reduction is dose-dependent.

Microscopic study of a large number of hairs exposed to x-rays on the 10th day (Figures 2-5) of anagen revealed that injury short of alopecia is almost inevitably followed by complete recovery (Figure 6). These changes occur rapidly, so that the techniques employed in the current study measure reduced growth rates that persist for only a few days during the 17-20 day period of anagen. The magnitude of damage to the affected part of the hair, then, is far greater than the experimental findings for whole hairs initially suggest, since only a small segment of the shaft is involved in these alterations. Micrometer measurements of hairs treated with 400-600 rads reveal that morphologic postirradiation narrowing and widening of the hair shaft only involve about 20 per cent of the hair's length. If it is assumed for illustrative purposes that hair

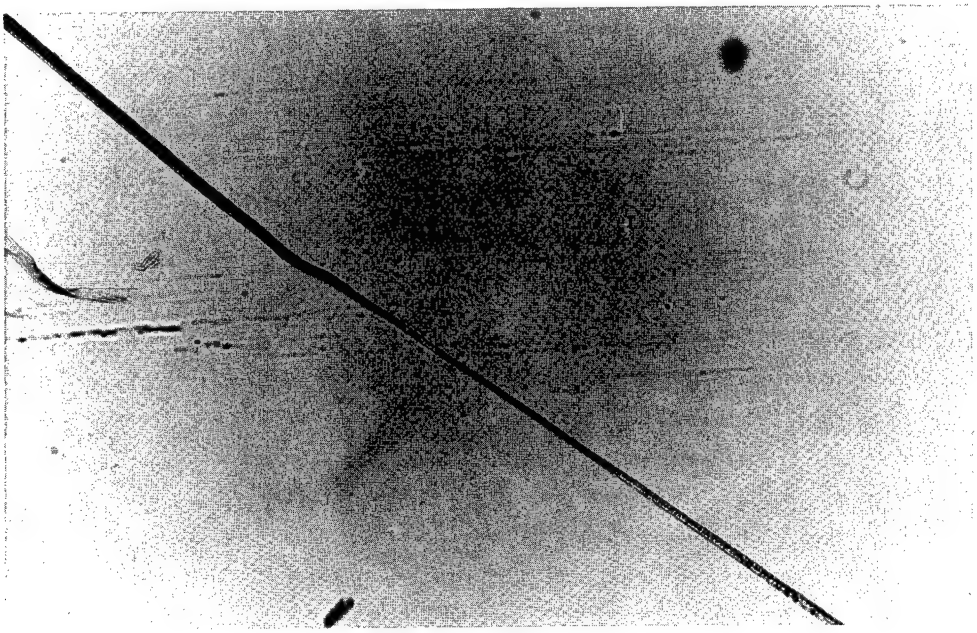

Figure 2. Irregularities and narrowing of the shaft in an anagen hair treated with 600 rads. Tip of hair is to the left. Some widening and recovery of shaft narrowing is present on the right (x 12.8).

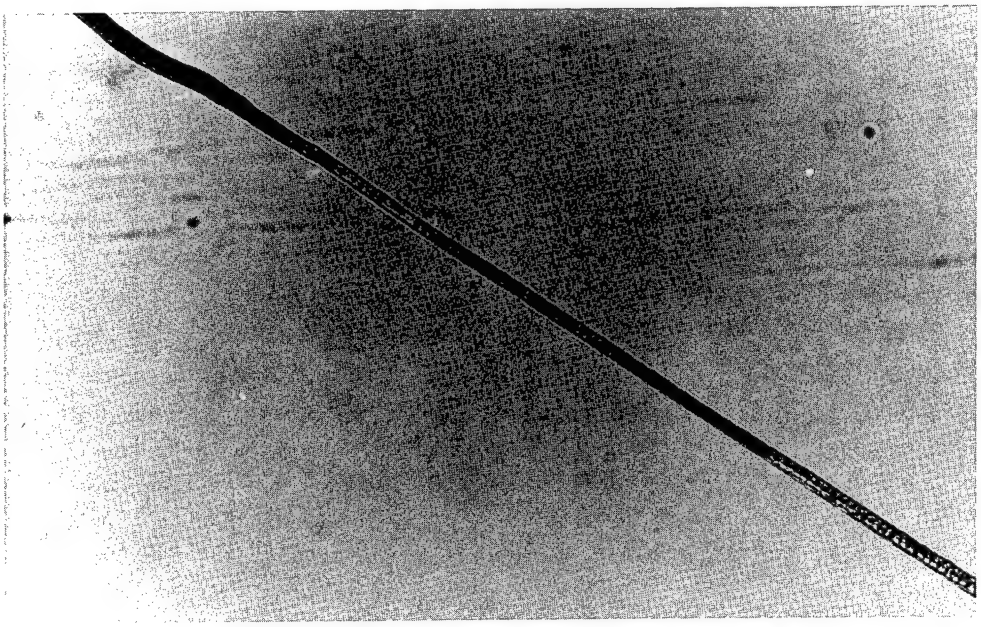

Figure 3. Same hair as Figure 2 (x 25.6).

growth rates are normally uniform throughout anagen (in actuality, some variation exists), then the average 8 mm hair must grow approximately 1.6 mm during each 20 per cent interval of the growth period. Consequently, the 11.7 per cent reduction in the entire hair length reflects an actual shortening of about 0.9 mm (see Table 1) for the affected hair segment, reducing the expected

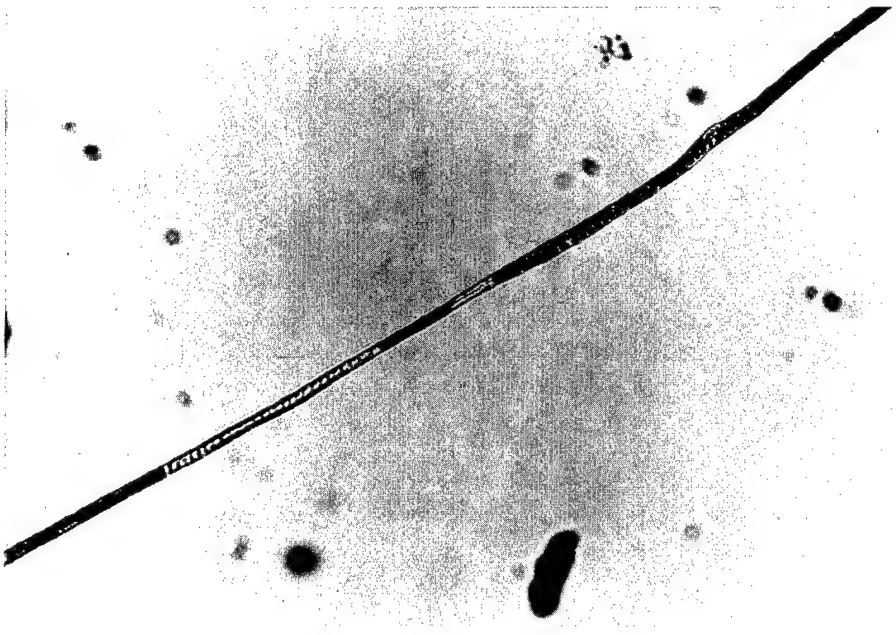

Figure 4. Anagen hair treated with 400 rads. Tip of hair is to the right (x 25.6).

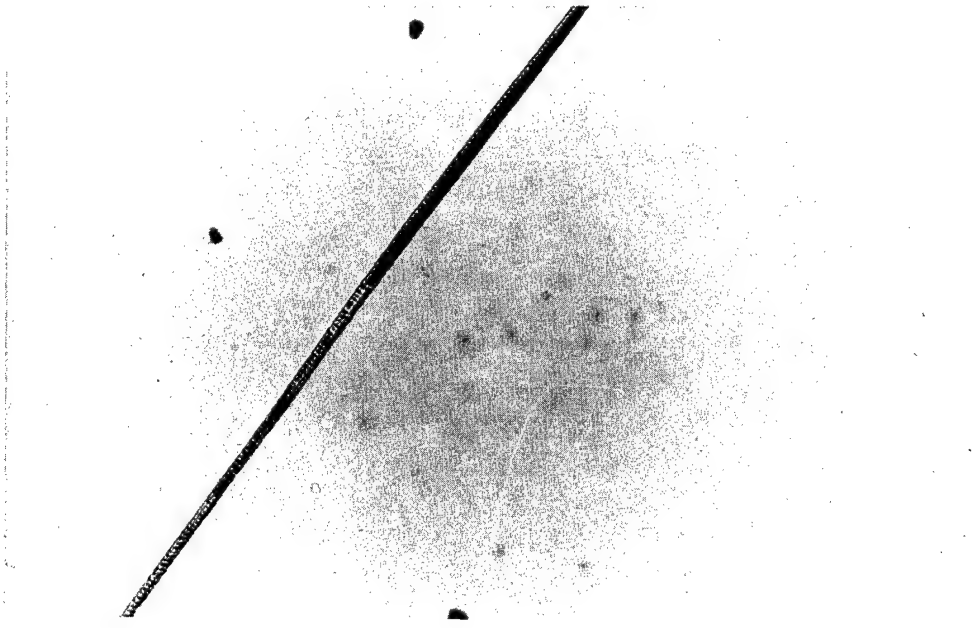

Figure 5. Anagen hair treated with 200 rads (x 25.6).

growth increment by well over 60 per cent. Similarly, in anagen hairs exposed to 200 rads, growth rates for the involved portion of the shaft are reduced over 40 per cent. These conclusions are based on the reasonable assumption that reduced growth rates and microscopic morphologic injury occur simultaneously, although this remains to be established.

The relatively small differences in overall retardation of hair growth with relatively large

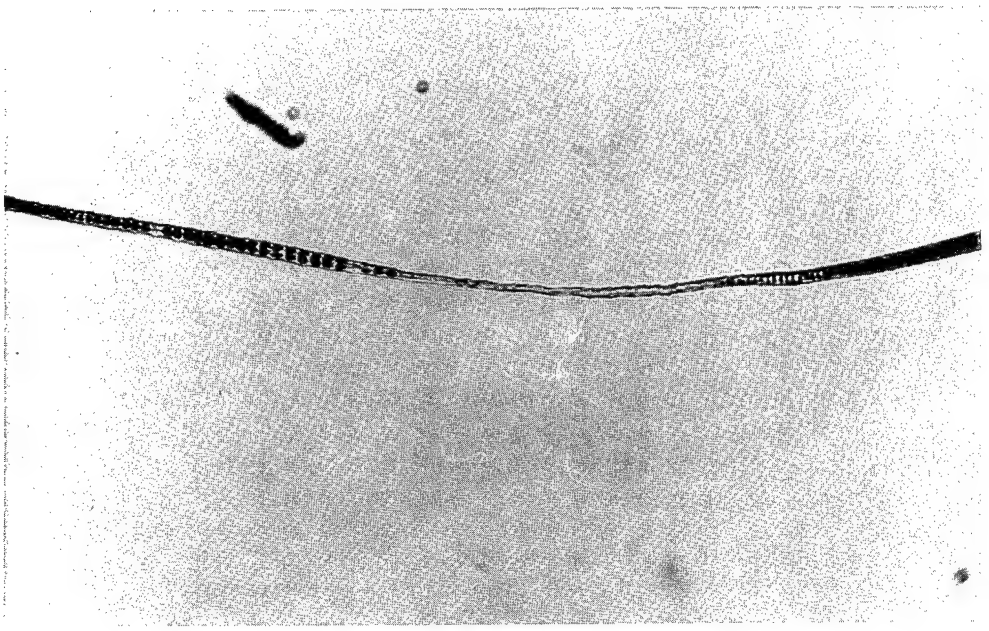

Figure 6. More severe damage in another anagen hair treated with 200 rads. Complete recovery from injury is seen. Tip of hair is to the right (x 25.6).

variations in dose suggest that measurements of markedly reduced growth rates in the affected segment of the hair alone should increase the usefulness of the method for further radiobiological investigations. Autoradiographic studies of the hair following administration of a series of ^{35}S-cystine injections concurrently with one or more radiation exposures should provide more sensitive indicators of dose differences. Measurements of shaft distances separating successive timed injections, together with observations on the rapidity of onset of depressed cystine uptake and quantitative differences in cystine incorporation, should all increase the sensitivity of the method. The possible use of such a technique is illustrated in Figure 7.

The establishment of radiation-induced dose-dependent alterations in hair growth rates provides a rapid, simple, non-destructive in vivo method that could be useful in the evaluation of a number of radiobiological phenomena. Varying effects of changes in dose fractionation schedules, of different modalities of radiation, and of depth-dose alterations (as for example, with electron beam therapy), are all problems that could be studied with this technique. Further, it might provide a suitable method for assay of radioprotective or radiosensitizing agents and for retrospective dose estimations following accidental radiation injury.

In the past, radiation responses have been studied far more intensively in mitotically active cells than in resting cell populations. The current studies demonstrate that non-dividing matrix cells are also susceptible to radiation damage. Much larger doses of radiation, however, are required to produce this effect. It is presumed that mitotically inactive telogen matrix cells are responsible for formation of the succeeding anagen hair. Since plucking is not followed by onset of matrix cell division until 72 hours later,[10] it is apparent that telogen matrix cells sustain and "store" radiation damage, which is then expressed following appropriate mitotic stimulus. Presumably radiation lesions in these cells are concerned primarily with effects on DNA synthesis and cellular division. Several intriguing questions arise concerning the persistence of "latent"

Figure 7. Autoradiograph of symmetrically contralateral rat vibrissae. Five successive intravenous injections of ^{35}S-cystine (10 μc per injection) were administered at 72-hour intervals. 1400 rads were given to the lower hair immediately preceding the 2nd injection. After 72 hours hair growth was slowed to about 80 per cent of that observed for the unirradiated hair. Growth retardation persisted for six days but some recovery is evident at the time of the 5th injection (x 5).

radiation damage in resting matrix cells, the speed of recovery from injury once cellular division is initiated, and the induction of more persistent chromosomal damage. The responsiveness of telogen matrix cells to radiation damage is also of interest in regard to recent observations of reduced mitosis in regenerating liver cells irradiated in the resting state prior to injury.[11,12]

Impaired growth of irradiated anagen hairs or of anagen hairs that have replaced irradiated telogen hairs must be related to cell population changes in the hair matrix. Most cell renewal systems depend on the continued production of cells, some of which divide again, while others progressively differentiate and mature. The primary effect of irradiation is to impair cell production, which leads to cell depletion, the degree of depletion depending on the magnitude of the impairment.[13] Differentiation may also be impaired, although effects on already matured hair matrix cells are probably far less critical. The hair matrix will survive as an organ only if a few remaining cells ultimately resume compensatory proliferation until repopulation occurs. It is obvious that the interrelationship between cell depletion and regeneration is extremely important to the survival of the irradiated matrix. During the depletion period it appears that only a fraction of the normal number of cells is required to perform the usual functions of the hair matrix, even though such functions may be carried out at a reduced rate. The number of cells undergoing differentiation and maturation under such circumstances probably remains proportional to

the size of the proliferative pool. Photomicographs of irradiated anagen hair enlarged 600-700 times permitted measurements which revealed that about two-thirds of the damaged hair segment showed progressive or sustained narrowing, with an abrupt widening to normal diameter in the remaining portion. Apparently, then, complete population recovery of residual hair matrix cells occurred abruptly within a period of 24-48 hours. The regeneration of depleted matrix cells could have been accomplished either by a shortening of cell cycle time and/or by temporary initial retention of most newly dividing cells in the proliferative pool with minimal loss of cells by differentiation and maturation.

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